

**Tissue-specific control of
macrophage phenotype and its
relevance to inflammation**

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**Thesis presented for the degree of
Doctor of Philosophy (Medicine)**

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Summary

Tissue resident macrophages are a highly heterogeneous cell type present in every mammalian tissue, where they have a critical role in innate immune defence and tissue homeostasis. Tissue-specific master regulators have been identified in different tissue-resident macrophage populations, which regulate macrophage tissue-specific phenotype and function through the induction and maintenance of specific transcriptional programs. However, it is largely unclear how transcriptional master regulators control tissue-specific macrophage phenotype during the initiation of the inflammatory response and the resolution thereof. Genetic deletion of these tissue-specific transcriptional regulators typically results in the ablation of the specific macrophage populations, such as the loss of splenic red pulp macrophages in *Spi-C* deficient mice. Conversely, genetic deletion of *Gata6* in macrophages does not completely ablate the population of peritoneal macrophages, albeit it leaves them at reduced numbers. Thus, *Gata6*-deficient pMφs provide a unique tool to investigate how tissue-specific transcription factors regulate macrophage function during homeostasis and in response to environmental stimuli, such as those encountered during inflammation. As such, the work presented in this thesis aimed to characterise the role that *Gata6* has during the inflammatory response of peritoneal macrophages, to identify mechanisms by which it regulates inflammatory processes, and to try and identify whether other GATA family members had analogous roles in other myeloid cells. Several key findings were generated during the studies presented in this thesis. *Gata6*-deficient macrophages were found to have dysregulated production of TNF, as well as the NLRP3- and caspase-1-dependent release of IL-1β in response to LPS stimulation alone. Interestingly, the aberrant release of pro-inflammatory cytokines from *Gata6*-deficient pMφs was attenuated by co-culturing with pMφs from *Gata6*-WT mice, suggesting that *Gata6* might release soluble factors that mediate the resolution of inflammation. I identified prostacyclin synthase to be highly expressed in resident pMφs and show that its expression was greatly reduced in *Gata6*-deficient pMφs. Consequently, the production of prostacyclin was severely diminished in *Gata6*-deficient pMφs following LPS stimulation compared to those from *Gata6*-WT mice. Treatment of LPS-stimulated *Gata6*-deficient pMφs with beraprost, a prostacyclin analogue, restored pro-inflammatory cytokine production comparable to pMφs from *Gata6*-WT mice. The transcription factor GATA3 was found to be

selectively up-regulated in Langerhans cells (LCs) generated in vitro from the MUTZ-3 cell line compared to the precursors. However, the knockdown of GATA3 expression with shRNAs did not affect the development, maintenance, or phenotype of LCs in this model.

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List of Abbreviations

ACK	Ammonium-Chloride-Potassium
ACKR	Atypical chemokine receptor
AGM	Aorta-gonad-mesonephros
AIM2	Absent in melanoma 2
AML	Acute myeloid leukaemia
AP1	Activator protein 1
APECs	Adherent Peritoneal Exudate cells
APS	Ammonium persulfate
ASC	Apoptosis-associated speck-like protein containing a CARD
ATF3	Activating transcription factor 3
BCA	Bicinchoninic acid
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone-marrow-derived macrophage
BSA	Bovine serum albumin
C1q	Complement component 1q
cAMP	Cyclic AMP
CARD	Caspase recruitment and activation domain
cDNA	Complimentary DNA
CLR	C-type lectin receptor
COPs	CARD-only proteins
COX	Cyclooxygenase

CREB	Cyclic AMP-responsive element binding protein
CTLD	C-type lectin-like domain
DAMP	Damage-associated molecular pattern
dH ₂ O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMP	Erythroid myeloid progenitor
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPCR	G-protein-coupled receptor
GSDMD	Gasdermin D
HEK 293T	Human Embryonic Kidney 293T
HSC	Hematopoietic stem cell
IAP	Inhibitor of apoptosis
IFN	Interferon
IL	Interleukin
IL-1RL1	IL-1 receptor-like 1
ILC	Innate lymphoid cell

iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
IRAK	IL-1R-associated kinase
I κ B	Inhibitor of NF- κ B
JNK	JUN N-terminal kinase
KO	Knock-out
LB	Lysogeny broth
LC	Langerhans Cell
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MBL	Mannose-binding lectin
MFG-E8	Milk-fat globule-EGF factor 8 protein
MLKL	Mixed lineage kinase like
MPS	Mononuclear phagocyte system
MSU	Monosodium urate
mt	Mitochondria
MyD88s	MyD88 short
NEMO	NF- κ B essential modulator
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLR	NOD-like receptor

NLS	Nuclear localisation sequence
NO	Nitric oxide
NOD	Nuclear oligomerization domain
NSAIDs	Non-steroidal anti-inflammatory drugs
O.D.	Optical density
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PCR	Polymerase chain reaction
PECs	Peritoneal Exudate Cells
PGE ₂	Prostaglandin E ₂
PGI ₂	Prostacyclin
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
pMφ	Peritoneal macrophage
POPs	PYD-only proteins
PPAR	Peroxisome proliferator-activator receptor
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PYD	Pyrin domain
qPCR	Quantitative Polymerase Chain Reaction
RA	Retinoic Acid
RHIM	RIP homotypic interaction motif
RIG-I	Retinoic acid-inducible gene I

RIN	RNA integrity number
RIPA	Radio-Immunoprecipitation Assay
RIPK	Receptor interacting protein kinase
RLR	RIG-I like receptor
ROS	Reactive oxygen species
RP105	Radioprotective 105
RPM	Red Pulp Macrophage
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SHP	Small heterodimer partner
shRNA	Small hairpin RNA
SOC	Super Optimal broth with Catabolite repression
SOCS	Suppressor of cytokine signalling
SP	Surfactant protein
SPF	Specific pathogen-free
SR	Scavenger receptor
sTLR	Soluble TLR
Syk	Spleen tyrosine kinase
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween® 20
TEMED	N,N,N',N'-Tetramethylethylenediamine

TF	Transcription Factor
TGF- β 2	Transforming Growth Factor- β 2
Th	T helper
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
TOLLIP	Toll-interacting protein
TRAF	TNF receptor-associated factors
TRAIL-R	Tumor necrosis factor-related apoptosis-inducing ligand receptor
TRIM	Tripartite-motif
TX	Thromboxane
UTR	Untranslated region
WR	Working reagent
WT	Wild-type
β ME	Beta-Mercaptoethanol

Chapter One

General Introduction

1.1 Macrophages

1.1.1 Historical perspective

Over a century ago, Élie Metschnikoff described the uptake of foreign material by specialised digestive cells in turbellarian worms (Metschnikoff, 1878). These observations provided the foundations for his seminal work showing the clearance of foreign particles from tissues by migratory devouring cells he named ‘phagocytes’ (from the Greek ‘phago’, meaning ‘devour’, and ‘cytos’ meaning ‘cell’)(Metschnikoff, 1884). Although the description of phagocytosis had been described earlier, reviewed by (Stossel, 1999), Metschnikoff’s seminal work and proceeding experiments demonstrated that phagocytosis was critical in the host response against infectious agents such as bacteria (Metschnikoff, 1902, Metschnikoff, 1888, Metschnikoff, 1887). He later categorized phagocytes as macrophages and microphages (now called neutrophils) in multiple organs and noted the cellular heterogeneity. Furthermore, he showed that macrophages are not only important for host cell defence but also as scavengers of degenerating host cells (Metschnikoff, 1887). Metschnikoff is considered to be the father of natural immunity, and his work formulated the theory of immunity by phagocytosis (Gordon, 2008).

1.1.2 Macrophage ontogeny

Over the last few years, the field of macrophage ontogeny has evolved rapidly. For decades, the prevailing dogma was that tissue-resident macrophage populations were derived from blood-circulating monocytes, arising from progenitors in the bone marrow (BM). This concept was the foundation of the ‘mononuclear phagocyte system’ (MPS), suggested by Van Furth and colleagues in the 1970s. This concept was supported by numerous studies which demonstrated: BM cells and monocytes could differentiate into macrophages *in vitro* and, adoptively transferred labelled monocytes migrated into tissue and differentiated into macrophages within tissues. However, this view has been challenged by the results of lineage-tracing studies, which indicate that embryonic precursors give rise to many tissue-resident macrophage populations (Figure 1.1). In adulthood, some of these tissue-resident macrophages such as microglia and Langerhans cells (LCs) maintain themselves largely independent of blood-borne monocytes, through longevity and self-renewal (Ginhoux et al., 2010, Schulz et al., 2012, Hashimoto et al., 2013, Yona et al., 2013). Conversely, some tissue-resident

macrophage populations such as those of the dermis and lamina propria are thought entirely derived from circulating monocytes (Bain et al., 2014, Varol et al., 2009). In the steady state, most other tissues are composed of a mixture of embryonically-derived and monocyte-derived macrophages, which varies between tissues. Recent studies indicate that monocyte-derived macrophages slowly repopulate some tissues and displace the embryonically-derived population. For instance, Ly6C⁺ monocytes were shown to constitutively enter the peritoneal cavity, where they matured and subsequently replaced the embryonic population with age. Interestingly, this process was highly gender specific and demonstrated that the embryonic population were not proliferatively exhausted (Bain et al., 2016). Furthermore, the monocyte-derived cells were found to acquire Gata6 and CD102, two markers of mature peritoneal macrophages (pMφs) in adult mice (Gautier et al., 2014, Okabe and Medzhitov, 2014, Rosas et al., 2014). However, these cells failed to express the phagocytic receptor Tim4, which has been shown to be expressed by resident pMφs. Thus, extensive transcriptional and functional studies are needed to determine whether monocyte-derived pMφs are capable of adopting the full phenotype and function of the embryonic cells they replace.

During the development of mammalian embryos, haematopoiesis consists of a tightly regulated stepwise process which begins in the yolk sac, passes through the fetal liver and eventually to the bone marrow, which is the location of haematopoiesis in adults (Figure 1.1). In developing mouse embryos, haematopoiesis begins in the yolk sac (primitive haematopoiesis), producing erythrocytes and macrophages. Primitive haematopoiesis is replaced by a transient wave of definitive haematopoiesis, which generates erythroid myeloid progenitors (EMPs) (McGrath et al., 2011, Bertrand et al., 2007). Furthermore, definitive haematopoiesis in the aorta-gonad-mesonephros (AGM) region in the embryonic mesoderm generates haematopoietic stem cells (HSCs), which populate the fetal liver at around embryonic day 10.5 (E10.5).

Thus, most tissue-resident macrophages develop locally and independently from those in other tissues, irrespective of their common lineage and embryonic-precursor origin. Furthermore, different tissues are composed of a mixture of embryonically-derived and monocyte-derived macrophages, for which recent studies suggest that in some tissues, monocyte-derived macrophages displace the embryonically-derived macrophages with age. However, it is currently unclear whether macrophages of different origin are functionally interchangeable and how this impacts tissue-resident macrophage identity and function at the steady state and during inflammation. Recent evidence suggests that environmental cues rather than ontogeny predominantly influence tissue-resident macrophage transcriptional programs and function

during the steady state (see 1.1.4 Macrophage transcription factors). However, whether BM-derived macrophages can recapitulate all functions and homeostatic features of the pre-existing embryonic macrophages they replace, especially in response to chronic inflammation, remains to be fully investigated.

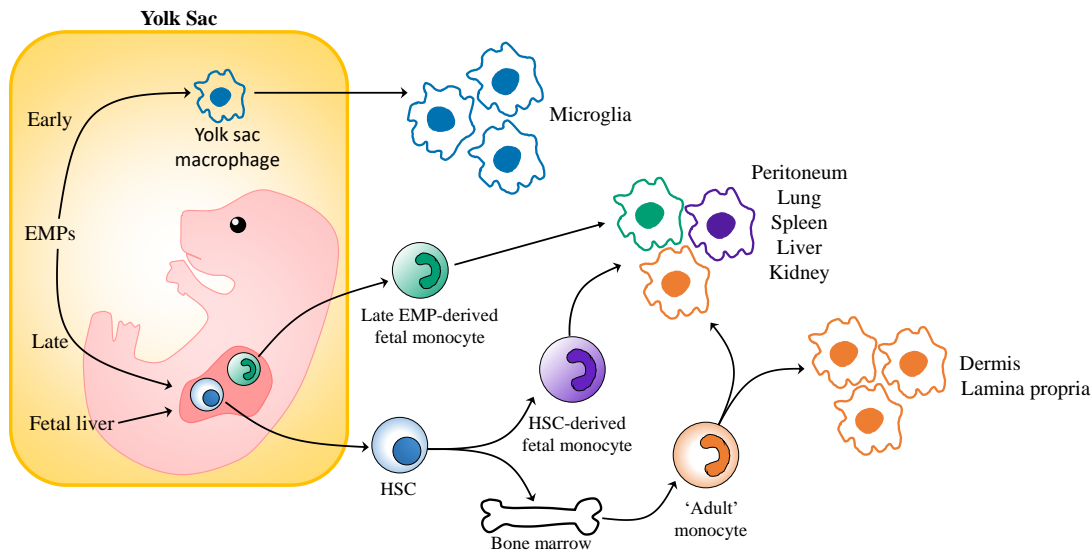


Figure 1.1 | Tissue-resident macrophage origin during steady state.

Most tissue-resident macrophage populations in the steady state arise from embryonic precursors (embryonic-macrophages) independently of BM-derived monocytes, except for dermal macrophages and intestinal macrophages. Fate-mapping studies suggest that embryonic macrophages derive from yolk sac-derived erythro-myeloid precursors (EMPs). A wave of EMPs appear around embryonic day 7.5 (E7.5) in the yolk sac, which colonise the brain and other fetal tissues around E9 (fetal macrophages). In parallel, definitive haematopoiesis occurs in the aorta–gonads–mesonephros (AGM) from E10.5, that gives rise to haematopoietic stem cells (HSCs), which colonise the fetal liver at around E10.5. Haematopoiesis in the fetal liver generates monocytes (fetal liver monocytes) around E11.5–E12.5, which migrate to embryonic tissues around E13.5–E14.5, where they largely replace the fetal macrophages, with the exception of microglia. A late wave of EMPs is thought to contribute to fetal liver haematopoiesis from E8.5 and may also contribute to the generation of fetal liver monocytes. During adulthood, tissue-resident macrophages are thought to be maintained through self-renewal and/or longevity (autonomous maintenance), as well as through conventional haematopoiesis. The requirement on blood monocytes for macrophage replenishment versus autonomous maintenance of tissue-resident macrophages is highly tissue-specific and is reported to be increasingly dependent on monocyte replenishment with age.

1.1.3 Macrophage function and development

Tissue-resident macrophages are a highly heterogeneous cell type, present in every mammalian tissue, where they play fundamental roles in development, homeostasis, tissue repair, and host defence (Gordon and Taylor, 2005, Wynn et al., 2013). In addition to these roles, macrophages perform numerous tissue-specific functions, depending on the tissue in which they reside (Davies et al., 2013, Gordon and Plüddemann, 2017). For instance, microglia, the brain-resident macrophages play a role in synaptic pruning and the clearance of debris. Red pulp macrophages in the spleen phagocytose senescent erythrocytes and recycle heme to maintain iron homeostasis. pMφs have been shown to regulate gut immunoglobulin (Ig) A production by interacting with peritoneal B-1 cells (Okabe and Medzhitov, 2014). Recently, tissue-resident pMφs were shown to rapidly migrate to the liver following sterile injury via a non-vasculature route, whereby they promote repair after hepatic death (Wang and Kubes, 2016). Interestingly, it has been known for some time that following intraperitoneal injection of bacterial components, pMφs “disappear”, a phenomenon commonly referred to as the “macrophage disappearance reaction” (Barth et al., 1995). This disappearance has been attributed to increased tissue adherence, cell death and tissue-emigration through draining lymphatics (Davies et al., 2011). Thus, these recent studies provide important functional insights into these earlier observations, whereby pMφs are thought to act as a reservoir of mature macrophages to promote tissue repair after acute injury.

Thus, despite common origins of most tissue-resident macrophages, each tissue-resident macrophage population performs distinct tissue-specific functions. Comprehensive transcriptional analysis of resident macrophage populations suggested macrophage populations from different organs exhibited considerable transcriptional diversity, underlying their tissue-specific roles (Gautier et al., 2012, Okabe and Medzhitov, 2014). These studies suggest that local factors within the environmental niche of each specific tissue-resident macrophage population might govern the regulation, maintenance, and functional specialisation of tissue-resident (Table 1.1). Indeed, earlier studies have demonstrated the importance of tissue-derived factors in the development and maintenance of macrophages. For instance, targeted ablation of colony stimulating factor 1 (CSF1) showed severe depletion of macrophages in many tissues including the skin, brain, bone, liver, and kidney (Cecchini et al., 1994). Furthermore, osteoporotic mice which have an inactivating mutation in *Csf1*, which encodes M-CSF (the cognate ligand of CSF1R), showed greatly diminished macrophage populations like those of CSF1R-deficient mice (Chitu and Stanley, 2006). However, the loss of M-CSF did not affect

LC (60% of wild-type mice) or microglia development to the extent of CSF1R loss, indicating that another ligand may be responsible for the maintenance or development of these populations (Witmer-Pack et al., 1993). IL-34, a stroma-derived cytokine was subsequently shown to bind to CSF-1R, suggesting that this alternative ligand may be responsible for the development of LCs and microglia (Lin et al., 2008). Accordingly, IL-34^{-/-} mice were selectively deficient of LCs and microglia, but had little impact on BM, splenic, and liver macrophages (Wang et al., 2012). Furthermore, LC development is acutely dependent on transforming growth factor-beta 1 (TGF- β 1), exemplified by the lack of epidermal LCs in TGF- β 1-deficient mice (Borkowski et al., 1996).

1.1.4 Macrophage transcription factors

Emerging evidence indicates that the phenotypic diversity and specialization of tissue-specific macrophages are governed by distinct transcriptional master regulators (Lavin et al., 2014). These tissue-specific factors are thought to co-ordinately regulate macrophage identity and function in tandem with lineage-specific transcription factors (Medzhitov and Horng, 2009). The coordinated action of these transcription factors specifies the tissue-specific gene expression of each tissue-resident macrophage population, allowing each subset to perform their niche specific functions (Figure 1.2). Furthermore, stimulus-induced transcription factors such as NF- κ B coordinate the expression of genes that regulate the inflammatory responses. Interestingly, the accessibility of binding sites is imparted by tissue- and lineage-specific transcription factors (tissue-conditioning), thereby regulating the action of stimulus-induced transcription factors (Gosselin et al., 2014, Lavin et al., 2014). The molecular mechanisms underlying tissue-imprinting by tissue- and lineage-specific transcription factors are now starting to be unravelled, for reviews see (Glass and Natoli, 2016, Amit et al., 2016).

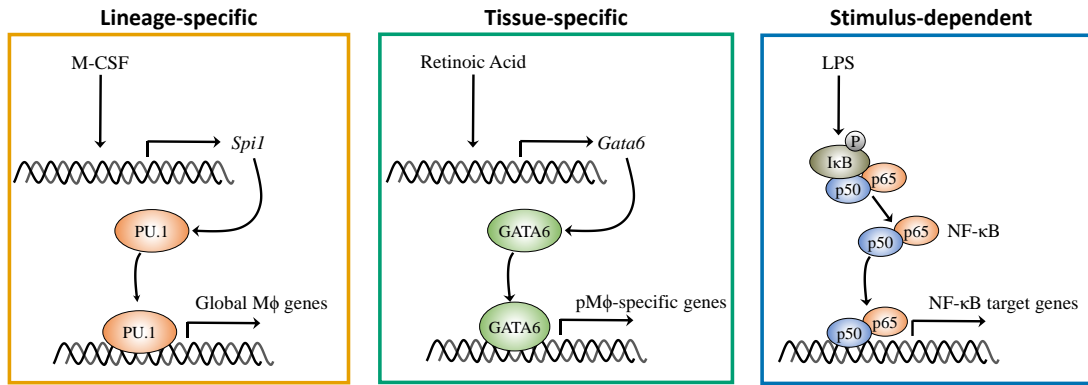


Figure 1.2 | Co-ordinate regulation of macrophage function and phenotype.

Lineage-specific transcription factors together with tissue-specific transcription factors (which are influenced by environmental factors) are thought to co-ordinately regulate macrophage identity and function. Stimulus-dependent transcription factors such as those activated by microbial stimuli regulate a plethora of genes that coordinate immune response. Recent evidence suggests that lineage- and that tissue-specific transcription factors regulate the accessibility of binding sites, which in turn influences the ability of stimulus-dependent transcription factors to elicit gene expression.

1.1.4.1 Lineage specific TFs

The ETS family transcription factor PU.1 is a critical regulator of hematopoietic development, particularly in the myeloid lineage. In mice, complete disruption of *Spi1*, the gene encoding PU.1, results in embryonic and/or new-born lethality. The critical dependence on PU.1 for macrophage development is exemplified in knockout models, which have an absence of circulating monocytes and mature tissue macrophages (Scott et al., 1994, McKercher et al., 1996, Iwasaki et al., 2005, Houston et al., 2007). PU.1 is further required by mature macrophages to respond to a variety of myeloid growth factors by modulating the expression of numerous genes, including those encoding M-CSFR and GM-CSFRα (Zhang et al., 1994, Zhang et al., 1996).

1.1.4.2 Tissue-specific TFs

Tissue-specific transcription factors have recently shown to play a critical role in determining the phenotype and function of the tissue-resident macrophages they control. Recent studies have identified and demonstrated the importance that tissue-specific transcription factors have on the phenotype and function of the tissue-resident macrophages they control (Table 1.1). For instance, the transcription factor Spi-C was found to be highly expressed in red pulp

macrophages and shown to be essential for their development; *Spic*^{-/-} mice show a selective loss of red pulp macrophages. Similarly, the transcription factor Gata6 was identified to be selectively expressed in peritoneal macrophages (Gautier et al., 2012). Conditional knockout of the *Gata6* gene in myeloid cells using the Cre-lox system (*Gata6*-KO^{mye}) revealed that whilst peritoneal macrophages were still present, they were greatly reduced in number, supporting a role for Gata6 in pMΦ persistence under homeostatic conditions (Rosas et al., 2014, Okabe and Medzhitov, 2014). The dramatic reduction in the number of pMΦs was attributed to the dramatic reduction in aspartoacylase expression (Gautier et al., 2014), however, other reports attribute the reduction of peritoneal macrophages to their accumulation in the omentum (Okabe and Medzhitov, 2014). Furthermore, the surviving *Gata6*-deficient pMΦs exhibited gross transcriptional defects in a large number of genes, many of which were peritoneal macrophage-specific genes (Rosas et al., 2014, Okabe and Medzhitov, 2014). As a result of these transcriptional defects, *Gata6*-deficient pMΦs were found to have defects in the control of IgA production by B-1 cells and defects in proliferative renewal during homeostasis and in response to inflammation (Okabe and Medzhitov, 2014, Rosas et al., 2014).

Table 1.1 | Tissue-resident macrophage populations, environmental cues, and their master regulators

Tissue-resident MΦ	Transcription factor/s	Environmental stimuli
Alveolar	Pparγ	CSF2
Intestinal	Runx3	CSF2
Kupffer	LxrA (ID3)	CSF1
Langerhans cell	Id2, Runx3	TGFβ, IL-34
Splenic Marginal zone	Lxra	Oxysterols, CSF1
Microglia	Sall1, Mef2C	TGFβ, IL-34, CFS1
Peritoneal	Gata6	CSF1, Retinoic acid, omental factors
Splenic Red Pulp	Spic	Haem, CSF2

Recent studies have demonstrated that these tissue-specific master regulators can be induced by stimuli from the tissue microenvironment. Retinoic acid (RA) was found to stimulate *Gata6* expression in peritoneal macrophages and therefore contributes to the tissue-specific transcriptional program (Okabe and Medzhitov, 2014). Similarly, *Spic* expression was found to be selectively induced in RPM development by heme, a metabolite of erythrocyte degradation (Haldar et al., 2014). Supporting a critical role of the microenvironment on imparting tissue-specific transcriptional programs, BM-precursors adoptively transferred into irradiated mice acquired 90% of the transcriptional profile of their embryonic-derived peritoneal and alveolar macrophages. However, this was tissue-specific as BM-derived Kupffer cells acquired less than 50% of the tissue-specific enhancers of their embryonic counterparts (Lavin et al., 2014). However, upon conditional deletion of embryonic Kupffer cells, one study showed that BM-derived Kupffer cells acquired an almost identical transcriptional profile to their embryonic counterparts (Scott et al., 2016). Furthermore, adult tissue-resident peritoneal macrophages transplanted into the alveolar cavity of donor animals acquired the chromatin landscapes and gene expression similar to alveolar macrophages, although the recovery of transplanted pMφs was low (Lavin et al., 2014). Overall, these results indicate that differentiated tissue-resident macrophages retain their plasticity and the critical importance of the tissue microenvironment on shaping the phenotype and function of macrophages (Lavin et al., 2014, Gosselin et al., 2014).

1.1.4.2.1 The GATA family of transcription factors

The GATA family of transcription factors are sequence-specific DNA-binding zinc finger proteins that are conserved in organisms from yeasts to mammals. The mammalian GATA family consists of six members, GATA1-GATA6, each of which displays a distinct and restricted pattern of tissue expression, exerting transcriptional control over a wide range of cellular processes. Members of the GATA family contain a highly-conserved DNA-binding domain composed of two adjacent Cys2/Cys2-type zinc finger motifs. The C-terminal zinc-finger and its adjacent highly-conserved basic region mediates sequence-specific DNA binding to the consensus DNA sequence (A/T)GATA(A/G). The N-terminal zinc finger is responsible for the specificity and stability of DNA-binding, but is also capable of binding DNA independently, with a preference for GATC core motifs. Both zinc fingers participate in binding the palindromic GATA motif ATC(A/T)GATAAG (GATApal), resulting in markedly increased affinity (Trainor et al., 1996).

1.1.5 Macrophage activation

In addition to lineage-specific and tissue-specific transcription factors determining macrophage phenotype and function, it has long been known that macrophages are able to adopt distinct functional phenotypes in response to extrinsic stimuli such as those from cytokines or microbial pathogens, which elicit dynamic changes in macrophage transcription, loosely known as macrophage activation (Gordon, 2003, Mosser, 2003, Benoit et al., 2008). Similar to lymphocyte activation, a dichotomous model for macrophage activation was proposed, the M1/M2 paradigm (Mills et al., 2000). These distinct phenotypes are induced by the actions of cytokines produced by two major classes of T lymphocyte populations: T helper 1 (Th1) and Th2 cells. Th1 cells produce interferon gamma (IFN- γ) whereas Th2 cells secrete interleukin 4 (IL-4) and IL-13 to promote the M1 and M2 activation states respectively (Nathan et al., 1983, Doyle et al., 1994, Stein et al., 1992). Broadly speaking, M1 macrophages (also referred to as classical macrophages) are typically described to have important roles in host defence against bacterial, viral, and protozoal pathogens (O'Shea and Murray, 2008, Mackaness, 1962, Gordon, 2007). They have enhanced antimicrobial, inflammatory and antigen-presenting properties, whereby they up-regulate and produce a plethora of pro-inflammatory mediators including pro-inflammatory cytokines and nitric oxide (NO). Conversely, M2 macrophages (also referred to as alternatively-activated macrophages) are generally described to have a critical role in the resolution of inflammation, producing an array of anti-inflammatory mediators (Gordon and Martinez, 2010, Mantovani et al., 2004, Martinez et al., 2008). Furthermore, M2 macrophages are thought to be critical in the defence against helminths, drive allergic responses, regulate wound healing, and drive fibrosis (Anthony et al., 2006, Murray and Wynn, 2011). The M1/M2 macrophage activation model has since formed the basis of research into macrophage activity ever since (Murray et al., 2014). The use of M1/M2 nomenclature has rapidly expanded, with the M1 designation restricted to classical activation, and M2 used to encompass various forms of macrophage activation other than M1 (Martinez et al., 2008). However, emerging studies have indicated that these macrophage populations may have marked differences in their physiology and biochemistry, both *in vitro* and *in vivo*. For instance, the M2 designation has been used to describe 'type-II activated macrophages', which like M2/alternatively activated macrophages, were shown to have reduced IL-12 secretion but higher levels of IL-10 production compared to M1 macrophages (Biswas et al., 2006, Mantovani et al., 2004). However, later studies have reported that type-II activated macrophages share more functional and biochemical similarities to M1 macrophages (Edwards et al., 2006). Similarly, one study

that analysed gene expression profiles of M2 populations elicited in a number of different murine disease models showed induction of a subset of genes in all populations irrespective of disease model, mouse strain, or source of cells (a common gene signature), they also had distinct differences in the expression of a large number of genes (Ghassabeh et al., 2006). Strikingly, further studies have reported that the phenotype and function of M2 macrophages induced by IL-4 stimulation were distinct in bone-marrow derived and tissue-resident macrophages (Gundra et al., 2014). Overall, it has become increasingly evident that the M1/M2 activation paradigm is too simplistic and rigid, and that macrophage activation most likely reflects a spectrum of functional states, which are both signal-dependent and plastic (Xue et al., 2014, Stout et al., 2005). Thus, a recent nomenclature system for macrophage activation that relates more specifically to the phenotype, function, and context of activation has been formally proposed to supersede the M1/M2 paradigm (Murray et al., 2014).

1.2 Inflammation

The cardinal signs of inflammation first described by Celcius have persisted into modern times, comprising *calor* (heat), *dolor* (pain), *rubor* (redness), and *tumor* (swelling). *Functio laesa* (loss of function) was later added to the description of inflammation by Galen. Inflammation forms the basis of many physiological and pathological processes. Inflammation is a fundamental protective response to the loss of cellular and tissue homeostasis, which is critical for host defence, the clearance of detrimental stimuli, and, tissue repair (Medzhitov, 2008). Inflammation is triggered by the recognition of conserved motifs on pathogens known as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), endogenous molecules which are released in response to tissue injury by germline-encoded pattern recognition receptors (PRRs) (see 1.3.1 Pattern recognition receptors) (Akira et al., 2006). PRRs are mainly expressed by myeloid cells including dendritic cells, macrophages, monocytes, and neutrophils, but are also expressed by epithelial cells, fibroblasts, and lymphocytes (Iwasaki and Medzhitov, 2004). Upon detecting PAMPs/DAMPs, PRR activation triggers the release of soluble factors including pro-inflammatory cytokines, chemokines, vasoactive amines, and lipid mediators including leukotrienes and eicosanoids. These soluble mediators have effects on local neighbouring cells (via autocrine and paracrine signalling), activating tissue-resident immune cells, as well as endothelial cells. The activation of endothelial cells increases vascular permeability and induces the expression of adhesion molecules such as VCAM-1 and ICAM-1. This allows the attachment of immune cells such as leukocytes to the endothelium and permit their subsequent transmigration into tissues at the site of infection/injury (Collins et al., 1995). Complement fragments C3a, C4a and C5a are produced by several pathways of complement activation, and promote the recruitment of monocytes and granulocytes, as well as inducing mast cell degranulation. The release of vasoactive amines such as histamine and serotonin by activated mast cells also serves to increase vascular permeability. When cytokines are released in larger quantities, they can exert endocrine effects such as the fever, fatigue, platelet activation, and the induction of acute-phase proteins in the liver, (Gruys et al., 2005). These acute-phase proteins produced by the liver serve to inhibit microbial growth by binding to iron (e.g., Ferritin), and enhance the clearance of microbial pathogens by acting as opsonins to promote phagocytic clearance (e.g., C-reactive protein and complement). Additionally, some acute-phase proteins produced by the liver such as serpins promote the resolution of inflammation. The release of endogenous lipid mediators by macrophages such as leukotrienes together with chemokines and cytokines serve as

chemoattractants, which promote the recruitment of immune effector cells such as neutrophils and monocytes to the site of infection/tissue injury (Bonecchi et al., 2009, Luster et al., 2005). The infiltration of effector cells is time-dependent, with neutrophils the first to enter the afflicted site, followed by monocytes (Serhan et al., 2007). Once at the afflicted site, neutrophils are activated either the recognition of PAMPs/DAMPs, or through the action of cytokines secreted by resident cells. Once activated, neutrophils produce a host of antimicrobial mediators to combat microbial pathogens including reactive oxygen and nitrogen species, as well as various proteinases. However, these potent effectors also damage host tissues, necessitating the need for the resolution of inflammation and the restoration of tissue homeostasis (Serhan and Savill, 2005). Historically, it was widely accepted that the cessation of inflammation was a passive process, where the loss of pro-inflammatory mediators would be considered the 'off signal'. However, it is now widely appreciated that the resolution of inflammation is an active and highly-regulated process, mediated by molecules that actively drive the termination of resolution, such as resolvins, prostaglandins and anti-inflammatory cytokines (Bannenberg et al., 2005, Serhan et al., 2000, Levy et al., 2001).

1.3 Innate Pathogen recognition

1.3.1 Pattern recognition receptors

Cells of the innate immune system express a large array of germline-encoded pattern recognition receptors (PRRs), which recognize and respond to a diverse array of highly-conserved structural motifs expressed by microbes known as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), endogenous molecules which are released in response to stress, tissue damage and necrotic cell death (Bianchi, 2007, Vance et al., 2009). The main functions of PRRs following pathogen recognition include phagocytosis, activation of pro-inflammatory signalling pathways, opsonisation, activation of the complement pathway, induction of apoptosis, and the activation of the adaptive immune response (Palm and Medzhitov, 2009, Gordon, 2002). PRRs can be defined as humoral (soluble) or cell-associated, with the latter group being further subdivided to cell surface and intracellular molecules (Figure 1.3).

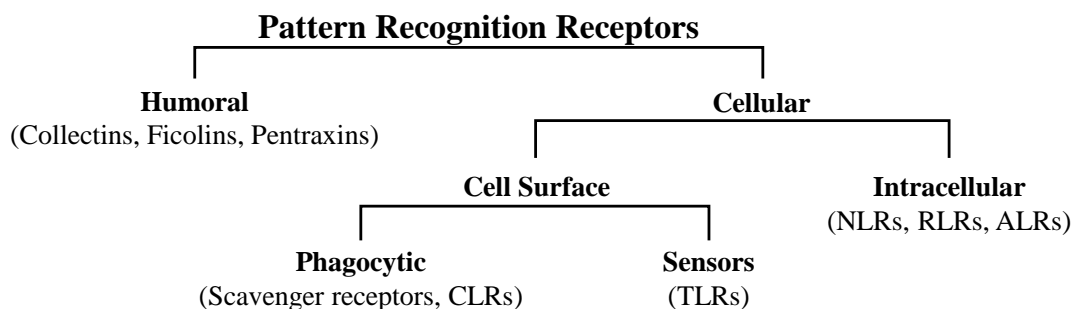


Figure 1.3 | Schematic representation of different groups of pattern recognition receptors. Abbreviations: ALR, AIM2-like receptor; CLR, C-type lectin receptor; NLR, NOD-like receptor; RLR, RIG-I-like receptor; TLR, Toll-like receptor.

1.3.1.1 Humoral PRRs

Humoral PRRs are key components of the innate immune response which include the collectins, ficolins, pentraxins, sCD14, milk fat globule-EGF factor 8 protein (MFG-E8), complement component 1q (C1q), and natural IgM. These receptors recognise PAMPs from a diverse array of pathogens of bacterial, fungal, and viral origin, as well as DAMPs on apoptotic, necrotic, and malignant cells (Rapaka et al., 2010, Palaniyar et al., 2004, Nauta et al., 2003).

The collectins (collagenous lectins) are a group of soluble PRRs belonging to the C-type lectin superfamily. The collectins, which include mannose-binding lectin (MBL), collectin 11, and surfactant protein A (SP-A) and D (SP-D) (Kawasaki et al., 1983, Haagsman et al., 1987, Benson et al., 1985, Hansen et al., 2010). The collectins share a similar structure consisting of an N-terminal collagen-like region which is involved in trimerization, and a ligand-binding C-type lectin domain at the C-terminus, which selectively binds to carbohydrate moieties including mannose, fucose, and N-acetylglucosamine (GlcNAc), which are prevalent on the surface of a wide array of bacteria, fungi, and viruses (Neth et al., 2000, Sastry and Ezekowitz, 1993, Holmskov and Jensenius, 1993, Drickamer, 1988). The ficolins (Ficolin-1, -2, -3) are another class of humoral PRRs that recognise N-acetylated carbohydrates present on microbial surfaces (Krarup et al., 2004). Ficolins consist of a collagen-like domain and a C-terminal fibrinogen-like domain that is responsible for carbohydrate recognition (Garlatti et al., 2007, Matsushita et al., 1996). Following recognition, humoral PRRs facilitate microbial clearance through complement activation, aggregation of microbial cells (agglutination), and opsonophagocytosis (Thiel, 2007, Endo et al., 2011, Ikeda et al., 1987, Matsushita et al., 1996). In addition, they also mediate apoptotic cell clearance and modulate inflammatory processes including cytokine production and chemotaxis (Schagat et al., 2001, Ogden et al., 2001, Taylor et al., 2000, Wright and Youmans, 1993, Gardai et al., 2003, Nadesalingam et al., 2005).

1.3.1.2 Cellular PRRs

Cellular PRRs encompasses a broad range of receptors and sensors that recognise a diverse array of PAMPs and DAMPs and can be divided into two groups based on their cellular localisation: cell surface or intracellular molecules. Cellular PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and Scavenger Receptors (SRs), which are transmembrane proteins which are located on the plasma membrane or endolysosomal membranes, whereas Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and absent in melanoma 2 (AIM2)-like receptors (ALRs) reside in the cytoplasm. Cell surface PRRs may be either phagocytic/endocytic receptors or sensor molecules. Phagocytic receptors directly bind to and internalise ligands. Conversely, sensor molecules do not directly internalise ligands, but instead initiate pro-inflammatory signalling cascades that culminate in antimicrobial effector responses.

1.3.1.2.1 Toll-like receptors (TLRs)

The Toll-like receptors (TLRs) are arguably the best-characterized group of PRRs (Kawasaki and Kawai, 2014, Beutler, 2009). To date, ten TLRs have been identified in humans and twelve in mice, with TLR1-TLR9 being conserved in both species, functional TLR10 present in humans (Mouse Tlr10 contains multiple sequence gaps and retroviral insertions), and TLR11, TLR12 and TLR13 present in mice but not humans. A number of genetic studies have demonstrated that different TLRs recognize distinct PAMPs and DAMPs in the extracellular space and in subcellular compartments including endosomes, lysosomes and endolysosomes (Table 1.2).

Table 1.2 | Toll-like receptors, cellular localisation, and their principal ligands

TLR	Species	Localisation	PAMPs	DAMPs
TLR1	Humans and mice	Plasma membrane	TLR1:TLR2: Triacyl lipopeptides	
TLR2	Humans and mice	Plasma membrane	TLR2: Lipoproteins TLR1:TLR2: Triacyl lipopeptides TLR2:TLR6: Diacyl lipoprotein, LTA, zymosan	Biglycan, endoplasmin, histones, HMGB1, HSP60, HSP70, MSU crystals, Versican
TLR3	Humans and mice	Endolysosomal compartment	dsRNA, tRNA, siRNA	mRNA
TLR4	Humans and mice	Plasma membrane and Endolysosomal compartment	Lipopolysaccharide, Mannan, Viral envelope proteins	Amyloid- β , Biglycan, β -defensin 2, endoplasmin, fibrinogen, fibronectin, heparin sulphate, histones, HMGB1, HSP22, HSP60, HSP70, HSP72, MSU crystals, oxidised low-density lipoprotein, S100 proteins, surfactant protein A
TLR5	Humans and mice	Plasma membrane	Flagellin	
TLR6	Humans and mice	Plasma membrane	TLR2:TLR6: Diacyl lipoproteins, LTA and zymosan	
TLR7	Humans and mice	Endolysosomal compartment	GU-rich ssRNA and short dsRNA	ssRNA

TLR8	Humans and mice	Endolysosomal compartment	ssRNA, Imidazoquinolones	ssRNA, Human cardiac myosin
TLR9	Humans and mice	Endolysosomal compartment	CpG DNA, hemozoin	Chromatin IgG complex, HMGB1, mtDNA
TLR10	Humans	Plasma membrane	Unknown –Evidence to suggest that TLR10 can form heterodimers with TLR1 and TLR2	
TLR11	Mice	Endolysosomal compartment	Profilin, flagellin, Uropathogenic bacteria	
TLR12	Mice	Endolysosomal compartment	Profilin	
TLR13	Mice	Endolysosomal compartment	Bacterial 23S rRNA with CGGAAAGACC motif	

Abbreviations: HMGB1, High-mobility group box 1 protein; HSP, Heat shock proteins; LTA, Lipoteichoic acid; MSU, Monosodium urate; mtDNA; Mitochondrial DNA

TLR receptors and signalling reviewed by (Kawasaki and Kawai, 2014, Beutler, 2009).

1.3.1.2.1.1 TLR signalling

Engagement of TLRs with their respective ligands stimulates downstream signalling pathways, culminating in the transcriptional upregulation of distinct genes, which is both TLR-specific and cell-type specific. TLR signalling can be broadly divided into two distinct pathways, depending on the usage of one of two signalling adaptor proteins, MyD88 and TRIF. All TLRs signal through MyD88 except for TLR3, which uses the adaptor TRIF. TLR4 is unique because it can signal through both MyD88 and TRIF adaptor proteins to perpetuate downstream signalling (Yamamoto et al., 2003). The activation of TLR signalling originates from the Toll/IL-1 receptor (TIR) domain, which is associated with the MyD88 adaptor. MyD88 recruits IL-1R-associated kinase (IRAK)-4 to TLRs, which activates other IRAK family members, IRAK-1 and IRAK-2 (Kawagoe et al., 2008). The IRAKs associate with the TNF receptor-associated factors (TRAFs), thereby activating the IKK complex, leading to the subsequent activation of Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the mitogen-activated protein kinases (MAPKs); p38 MAPK and JUN N-terminal kinase (JNK). Activation of the MAPK cascade leads to the activation of two transcription factor complexes; activator protein 1 (AP1) and cyclic AMP-responsive element binding proteins (CREB) (Figure 1.4).

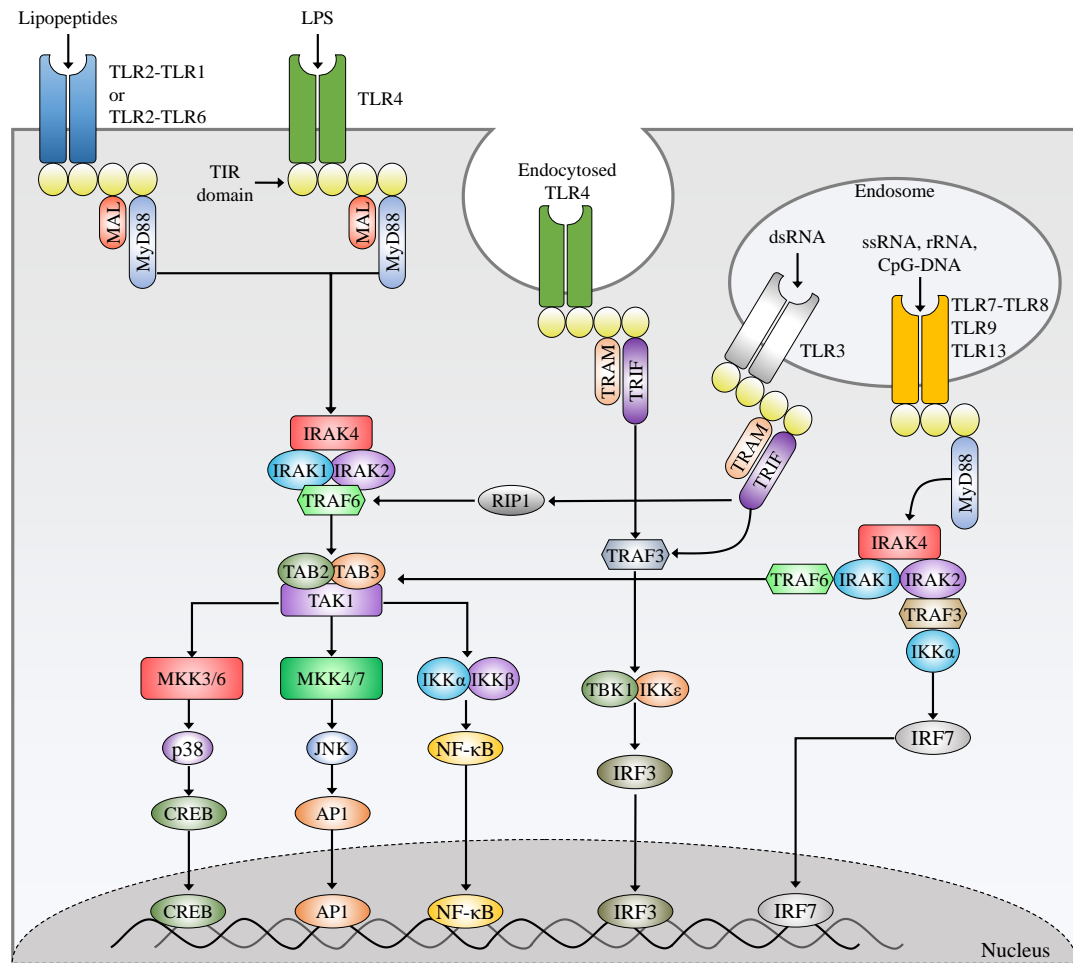


Figure 1.4 | Mammalian TLR signalling pathways.

TLRs signal through MyD88-dependent and MyD88-independent pathways. Upon respective ligand binding at the plasma membrane (TLR2/1, TLR2/6, TLR4/MD-2, and TLR5) or endocytic compartments (TLR7, TLR8, TLR9, and TLR13) recruit MyD88 to their TIR domains. TLR2/1, TLR2/6, and TLR4/MD-2 also recruit the adaptor protein MAL. MyD88 recruits IRAK proteins 1, 2 and 4, which recruit TRAF6 to the complex. The complex serves as a platform to recruit TAK1, which mediates the activation of the IKK complex, leading to the subsequent activation of NF- κ B and MAPKs (p38 and JNK). Activation of the MAPK cascade leads to the activation of two transcription factor complexes, AP1 and CREB. Conversely, TLR3 signalling is mediated through the TRIF-signalling pathway, which recruits TRIF and TRAM upon ligand binding. TRIF interacts with TRAF3, which activates TBK1 and IKK ϵ , resulting in the activation of IRF3. IRF3 translocates into the nucleus, whereby it activates the transcription of genes including type I IFN. TLR4 is unique because it can signal through both MyD88- and TRIF-dependent pathways, requiring endocytosis for TRIF-dependent signalling.

1.3.1.2.1.2 Negative regulation of TLR signalling

Dysregulation of TLR signalling can have severe consequences such as the cytokine storm associated with septic shock, autoimmunity, and lethality from ineffective defences against pathogens (Poltorak et al., 1998, Zacharowski et al., 2006, Stack et al., 2005, Roelofs et al., 2005, Leadbetter et al., 2002). Accordingly, a number of molecules that serve to negatively regulate TLR signalling have been identified, which have evolved to attenuate TLR signalling and prevent the deleterious effects of an overactive or prolonged innate immune response (Liew et al., 2005). Negative regulation of TLR signalling is achieved at multiple levels, ranging from soluble decoy receptors, membrane-associated protein regulators, regulation of adaptor complexes and signalling, as well transcriptional, post-transcriptional, and post-translational modulation, extensively reviewed by (Anwar et al., 2013, O'Neill et al., 2011, Murray and Smale, 2012, Kondo et al., 2012, Liew et al., 2005). Whilst some molecules that negatively regulate TLR signalling are constitutively expressed, most are induced downstream of TLR signalling, thereby attenuating TLR signalling in a negative feedback manner. Interestingly, mice deficient of constitutively expressed negative regulators of TLR signalling display spontaneous inflammatory or autoimmune diseases, suggesting that these molecules are important for controlling basal inflammatory responses to endogenous ligands. Soluble TLRs in blood and tissues, such as sTLR2 and sTLR4 have been reported to negatively regulate TLR signalling by functioning as decoy receptors, preventing direct interaction between bacterial ligands and cell-associated TLRs (Iwami et al., 2000, LeBouder et al., 2003). The regulation of TLR expression represents another mechanism through which TLR signalling is negatively regulated. TLR expression can be both downregulated by the actions of anti-inflammatory cytokines such as IL-10 and TGF- β 1 which inhibit the transcription or stability of TLR-encoding mRNAs, or through the degradation of TLR proteins via ubiquitination, which promotes the proteasomal degradation of TLRs (Chuang and Ulevitch, 2004, McCartney-Francis et al., 2004, Muzio et al., 2000). In addition to soluble factors, a number of transmembrane receptors have been implicated in the negative regulation of TLR signalling, including IL-1 receptor-like 1 (IL1RL1, also known as T1 and ST2), single immunoglobulin IL-1R-related molecule (SIGIRR), radioprotective 105 (RP105), and tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAIL-R) (Brint et al., 2004, Wald et al., 2003, Diehl et al., 2004). These molecules can negatively regulate TLR signalling through the

sequestration of transcription factors and adaptor proteins, or by interfering with the binding of TLR ligands to their respective TLRs. Following TLR:ligand ligation, TLR signalling can be further regulated by several intracellular negative regulators which include MyD88 short (MyD88s), IL-1 receptor-associated kinase M (IRAK-M), suppressor of cytokine signalling 1 (SOCS1), NOD2, phosphatidylinositol 3-kinase (PI3K), Toll-interacting protein (TOLLIP), A20, and activating transcription factor 3 (ATF3) (Watanabe et al., 2004, Zhang and Ghosh, 2002, Boone et al., 2004, Gilchrist et al., 2006, Whitmore et al., 2007). SOCS1 is a member of the E3 ubiquitin ligase family, which negatively regulates TLR signalling through the targeted degradation of MAL and TRAF proteins (Yoshimura et al., 2007, Mansell et al., 2006). The IRAK family member, IRAK-M, lacks intrinsic kinase activity and negatively regulates TLR signalling by preventing the dissociation of IRAK4 and IRAK1 from MyD88. As such, IRAK-M deficient mice secrete more pro-inflammatory cytokines in response to various TLR ligands compared to their wild type counterparts (Kobayashi et al., 2002). ATF3, a member of the ATF/CREB family of transcription factors, is induced following LPS stimulation and serves as a negative regulator of TLR signalling. Accordingly, ATF3-deficient macrophages have enhanced pro-inflammatory cytokine production (Gilchrist et al., 2006). ATF3 dampens TLR signalling by recruiting histone deacetylases, which alter the chromatin structure in the promoter region of genes encoding IL-6 and IL-12, restricting access for NF- κ B and AP1 transcription factors and thereby repressing expression (Whitmore et al., 2007). Overall, TLR expression and signalling are tightly controlled by numerous negative regulators, which serve to terminate inflammatory responses and prevent excessive inflammation.

1.3.1.2.2 C-Type Lectin Receptors (CLRs)

The term ‘C-type lectin’ was introduced to distinguish between Ca^{2+} -dependent (CLRs) and Ca^{2+} -independent animal lectins (Zelensky and Gready, 2005). The CLRs comprise a large family of soluble and transmembrane proteins that are defined by the presence of one or more C-type carbohydrate recognition domains (CRDs), a compact structural module that confers the carbohydrate recognition specificity of CLRs (Weis and Drickamer, 1996). The CLR family now includes members that contain one or more domains homologous to CRDs, but which do not bind to carbohydrates and are not calcium-dependent. Thus, “C-type lectin-like domain” (CTLD) is generally used

to refer to the structural module and “C-type lectin” consequently used to define proteins that contain one or more CTLDs, irrespective of carbohydrate recognition and dependency of calcium for binding. The CLRs encompass over 1000 members with diverse functions including cell adhesion, tissue remodelling, complement activation, platelet activation, regulation of natural killer function, endocytosis, phagocytosis, and activation of innate immunity (Weis and Drickamer, 1996, Osorio and Reis e Sousa, 2011, Zelensky and Gready, 2005).

The CLRs are highly expressed on myeloid cells where they have traditionally been associated with the recognition of fungi, but are now known to recognise a diverse array of microbial pathogens including viruses, mycobacteria, bacteria, and helminths (Rothfuchs et al., 2007, van Kooyk and Rabinovich, 2008, Robinson et al., 2006, Geijtenbeek and Gringhuis, 2009). Many of the PAMPs recognised by CLRs are carbohydrates: glucan structures present on mycobacteria and fungi; high-mannose structures expressed by fungi, mycobacteria, and viruses; and fucose structures on the surface of some bacteria and helminths. Following pathogen recognition CLRs elicit diverse signalling pathways, which is predominantly dependent on the signalling motifs in the cytoplasmic tails. Some CLRs, such as dectin-1, dectin-2 and mincle, induce signalling pathways that directly activate NF- κ B (Hara et al., 2007). Conversely, other CLRs including DC-SIGN, DCIR and MICL, induce signalling pathways that modulate TLR-dependent gene expression, at both the transcriptional and post-transcriptional level.

The phagocytosis of pathogens and their subsequent degradation in acidified phagolysosomes is an important mechanism of innate immune defence, recently reviewed by (Gordon, 2016). Whilst TLRs have not been shown to phagocytose pathogens, some studies have suggested that TLR signalling has a critical role in phagosomal maturation (Blander and Medzhitov, 2004). However, other studies have shown that phagosomal maturation is independent of TLR signalling (Yates and Russell, 2005). In contrast, many CLRs act as phagocytic receptors in myeloid cells, which upon recognition, internalise and subsequently degrade microbial pathogens. Distinct internalisation motifs are often contained within the cytoplasmic tails of CLRs, which both direct ligand uptake and subsequent sorting of receptors and cargo to endosomal compartments (Bonifacino and Dell'Angelica, 1999).

Dectin-1 is a transmembrane receptor that is highly expressed on myeloid cells (Taylor et al., 2002a), and is of particular relevance to this thesis. Dectin-1 is the major receptor for β -glucan, carbohydrate polymers found primarily in the cell walls fungi such as *Saccharomyces cerevisiae* and *Candida albicans* (Brown and Gordon, 2001). Zymosan is a β -glucan-rich preparation of cell wall from *Saccharomyces cerevisiae* that is recognised through receptors including, dectin-1, dectin-2, SIGNR1, mannose receptor, and TLR2/6 (Gantner et al., 2003, Brown and Gordon, 2001, Giaimis et al., 1993, Taylor et al., 2004, McGreal et al., 2006). Recognition of β -glucan containing fungal organisms and yeast-derived particles (such as zymosan) by dectin-1 leads to phagocytosis, which is mediated through the ITAM-like motif in the cytoplasmic tail of dectin-1 (Herre et al., 2004). A salient feature of PRRs is their ability to couple microbial recognition to the activation of downstream signalling pathways that mediate innate responses, such as the expression of pro-inflammatory cytokines. Dectin-1 has been definitively demonstrated to stimulate cytokine production, which is dependent on spleen tyrosine kinase (Syk) (Rogers et al., 2005). Mutagenesis studies suggest that Syk recruitment is mediated through a single YxxL motif in dectin-1 (where “x” is any amino acid). Once activated, Syk mediates the activation of the NF- κ B and the Erk, JNK, and p38 kinase cascades (Figure 1.5) (Robinson et al., 2006, Gross et al., 2006). Furthermore, dectin-1 and TLRs synergise in a Syk-dependent manner to produce optimal cytokine responses in macrophages (Dennehy et al., 2008), supporting a role for the recognition of pathogens simultaneously through different PRRs to control infections.

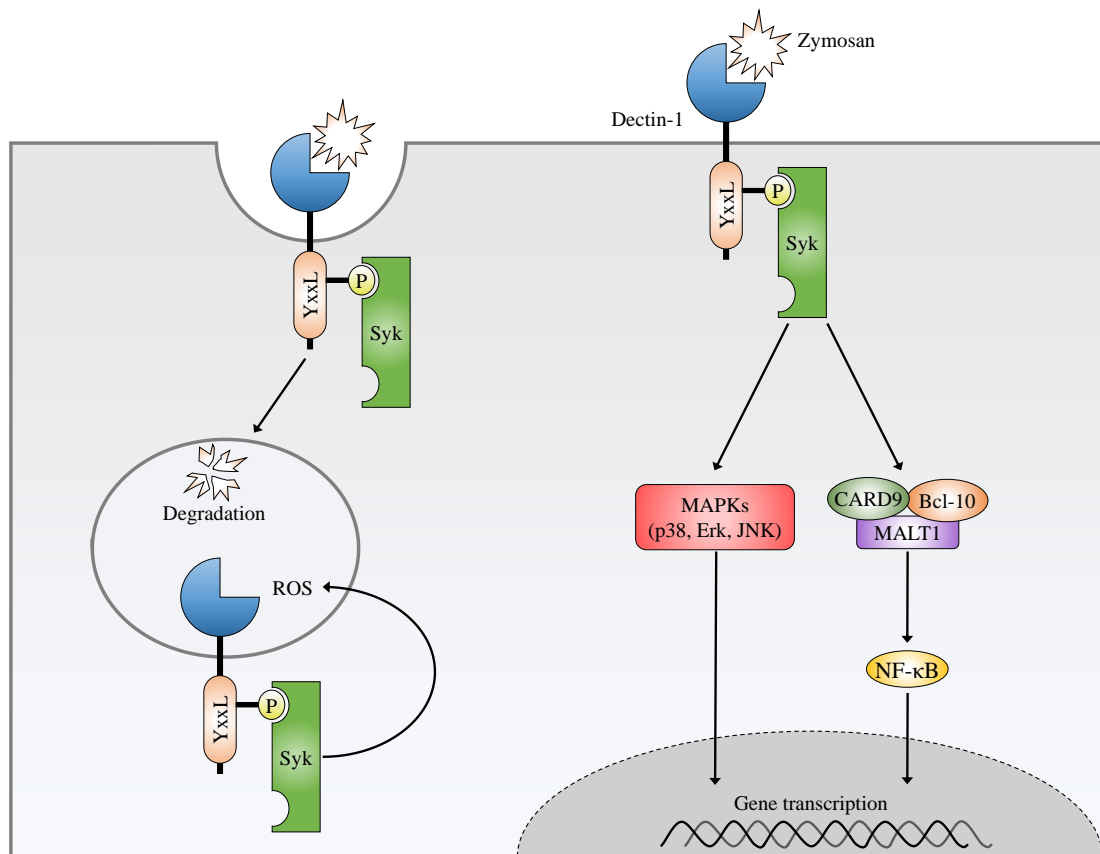


Figure 1.5 | Dectin-1 signalling.

Zymosan stimulation initiates the activation of MAPKs and NF-κB (the latter via a complex containing CARD9, Bcl-10 and MALT1) in a Syk-dependent manner. The activation of these transcription factors leads to the expression of innate immune responsive genes such as pro-inflammatory cytokines. Zymosan is phagocytosed and subsequently degraded in phagolysosomes. The internalisation of zymosan in macrophages is independent of Syk activation, but the production of ROS is Syk-dependent.

1.3.1.2.3 NLRs

The nucleotide-binding oligomerization domain receptors (NOD-like receptors, NLRs) are highly conserved cytosolic sensors of PAMPs and DAMPs. To date, twenty-two human and thirty-four mouse NLR family members have been identified (Kim et al., 2016b, Franchi et al., 2009). With few exceptions, NLR family members typically have a tripartite structure with a C-terminal leucine-rich-repeat (LRR) domain, a central NACHT domain, and an N-terminal caspase recruitment and activation (CARD) or pyrin (PYD) domain. The NLRs include NOD-1 and NOD-2, which recognise distinct ligands. NOD-1 recognises dipeptide γ -D-glutamyl-meso-diaminopimelic acid, whereas NOD-2 detects muramyl dipeptide, a component of

peptidoglycan (Girardin et al., 2003a, Girardin et al., 2003b, Inohara et al., 2001). Upon ligand recognition by NOD-1 and NOD-2, signalling cascades activate NF- κ B and MAPKs, which culminates in the production of pro-inflammatory cytokines. Some NLR family members oligomerise to form multimeric protein complexes called inflammasomes, following their activation by PAMPs or DAMPS (see 1.5 Inflammasomes).

1.3.1.2.4 Scavenger receptors (SRs)

The Scavenger receptors are a diverse group of PRRs that were originally identified by their ability to recognise and remove modified lipoproteins. SRs have distinct but overlapping recognition of a diverse array of PAMPs and DAMPs, as well as apoptotic ligands. These promiscuous receptors include SR-A, MARCO, and CD36, and bind polyanionic ligands (Canton et al., 2013). Overall, SRs identify and remove unwanted entities such as apoptotic cells, damaged proteins, and a variety of microbial pathogens (Mukhopadhyay and Gordon, 2004).

1.3.2 Phagocytosis

Phagocytosis is a remarkably complex process that is critical in development, tissue homeostasis, and immunity, extensively reviewed in (Flannagan et al., 2012, Gordon, 2016, Pauwels et al., 2017). Phagocytosis is the process by which cells recognise and internalise a variety of particulates ($>0.5\ \mu\text{m}$) into membrane-bound vesicles known as phagosomes, including microorganisms, environmental debris, and altered-self such as apoptotic, necrotic, and senescent cells (Penberthy and Ravichandran, 2016). Phagocytes can be grouped into ‘professional’ and ‘non-professional’ phagocytes. The professional phagocytes include macrophages, mast cells, monocytes, and neutrophils. Non-professional phagocytes include epithelial cells, endothelial cells, fibroblasts, and mesenchymal cells, which are capable of phagocytosing during development and some pathological circumstances and can therefore be considered facultative phagocytes (Gordon, 2016). Furthermore, non-professional phagocytes typically phagocytose a smaller variety of particulates and have a lower efficacy for internalisation than professional phagocytes.

Phagocytosis can be broadly broken down into a series of defined steps: recognition and binding of the target particle; actin-dependent ingestion of the target into a phagosome; the maturation of the phagosome into a phagolysosome via a series of

membrane fusion and fission events; and the degradation of the ingested particle in the acidic phagolysosome. However, the precise mechanisms of phagocytosis are remarkably heterogeneous, owing to the various combinations of receptors that recognise and internalise particulates, as well as the nature of the particle, which may influence the maturation rate of phagosomes (Erwig et al., 2006). For example, phagocytosis mediated by Fc γ receptors (Fc γ Rs) initiates 'reaching' phagocytosis that is typically characterised by membrane protrusions that extend to encapsulate the particulate, whereas particles internalised via complement receptors (CRs) are internalised without significant membrane protrusion, which is often referred to as 'sinking' phagocytosis (Underhill and Goodridge, 2012).

The Macrophage is considered the archetypal phagocytic cell; indeed, their name derives from the Greek for 'big eaters', owing to their phagocytic propensity. The phagocytic process is initiated by the recognition of particulates by plasma membrane receptors. The ability of macrophages to recognise, engage, and internalise a broad array of particulates therefore necessitates a vast repertoire of receptors and sensors (Freeman and Grinstein, 2014). Multiple receptors are co-expressed in single phagocytes, which collaborate in the detection and ingestion of particles. Furthermore, the activation of phagocytic receptors is generally dependent on lateral clustering, in which multivalent ligands on the surface of particles engage with multiple receptors on the phagocyte, which initiate cytoskeletal rearrangements and membrane trafficking leading to engulfment of the particulate (Gordon, 2016).

Particulates may be recognised directly by non-opsonic receptors including CLRs (mannose receptor, dectin-1), integrins (e.g., CR3), and SRs. However, in many instances recognition of particulates is mediated by opsonins such as iC3b, immunoglobulin G (IgG), and MBL, which coat the particulates and are themselves recognised by cell surface receptors. These cell surface receptors are generally referred to as the opsonic receptors, of which CRs and Fc γ Rs are the best characterised (Unkeless et al., 1988). CR3 has also been reported to mediate the recognition of particulates in the absence of opsonins, which is thought to be through macrophage-derived complement deposition (Le Cabec et al., 2002).

Although several types of receptor have been demonstrated to have roles in the phagocytic uptake of target particles, the definition of a bona fide phagocytic receptor

is complex. Only a few receptors have been reproducibly shown to be sufficient for phagocytosis upon ectopic heterologous in an otherwise non-phagocytic cell type. These include the Fc γ Rs and dectin-1, which recognise antibody-opsonised particles and fungal β -glucan respectively (Brown and Gordon, 2001, Indik et al., 1991).

However, whilst some receptors are unable to initiate target internalisation alone, a receptor that is necessary for the efficient binding of a particulate also participates in phagocytosis. A two-step model has been proposed to distinguish between receptors that 'tether' the particulate to the phagocyte, and receptors that provide a 'tickle' signal resulting internalisation of phagocytic targets: the 'tether and tickle' model (Hoffmann et al., 2001). Other receptors such as the TLRs may enhance phagocytosis by inducing the expression of phagocytic receptors such as Fc γ Rs, as well as the inducing inside-out activation of phagocytic integrins (Sendide et al., 2005).

1.3.3 Innate signalling pathways

The recognition of microbial pathogens and the initiation of downstream effector functions are linked through the activation of transcription factors such as NF- κ B, IRFs and MAPKs.

1.3.3.1 NF- κ B

The NF- κ B family of transcription factors have fundamental roles in innate immunity inflammation, cell proliferation, differentiation, and survival (Bonizzi and Karin, 2004, Caamano and Hunter, 2002). Since its discovery in activated B cells in 1986, NF- κ B has been found to be expressed in almost all cell types and tissues, and NF- κ B binding sites (κ B sites) are present in the promoters of a large number of genes. The NF- κ B pathway has long been considered the prototypical proinflammatory signalling pathway, largely because the recognition of microbial products, antigens and cytokines lead to the activation of NF- κ B. Furthermore, NF- κ B induces the transcription of pro-inflammatory cytokines, chemokines, and adhesion molecules, exemplifying its critical role in the innate and adaptive immune responses (Hayden and Ghosh, 2012). However, it has become increasingly evident that NF- κ B signalling is critical for the resolution of inflammation, whereby it induces the expression of anti-inflammatory cytokines, pro-resolvins and induces apoptosis (Lawrence, 2009, Greten et al., 2007).

In mammals, the NF- κ B family is composed of five members; NF- κ B1, NF- κ B2, RelA (p65), c-Rel and RelB. In contrast to the other family members, NF- κ B1 and NF- κ B2 are synthesized as pro-forms (p105 and p100), which are proteolytically processed to p50 and p52, respectively. These subunits form homodimer and heterodimers in the cytosol, which are determined by tissue-specific expression of different NF- κ B subunits (Hayden and Ghosh, 2004). The NF- κ B subunits all share a common 300 amino acid long Rel homology domain (RHD), which is required for dimerization, nuclear translocation, and DNA-binding (Ghosh et al., 1995, Chen et al., 1998, Muller et al., 1995). However, only p65, c-Rel and RelB contain carboxy-terminal transactivation domains (TAD). Thus, not all NF- κ B dimers are transcriptionally active, with some dimers (p50 and p52 homo- and hetero-dimers) demonstrated to repress κ B-dependent transcription (Zhong et al., 2002).

1.3.3.1.1 NF- κ B Signalling

In resting macrophages, NF- κ B dimers are retained in an inactive form in the cytosol through their interaction with one of seven inhibitor of NF- κ B (I κ B) family members. The binding of I κ B family members to NF- κ B dimers typically occludes the nuclear localization sequences (NLS) of NF- κ B subunits, thereby preventing nuclear translocation and subsequent induction of gene expression (Beg et al., 1992). Accordingly, the activation of NF- κ B is dependent on the degradation of I κ B proteins bound to NF- κ B dimers. However, more recent findings suggest that distinct I κ B family members exert different functional characteristics on NF- κ B responses, which is dependent on tissue-specific expression of I κ B proteins and different cellular contexts (Oeckinghaus and Ghosh, 2009).

Currently, two signalling pathways that lead to the activation of NF- κ B have been described; the classical (canonical) and the alternative (non-canonical) pathway (Figure 1.6). The ‘canonical’ pathway is activated by microbial products, pro inflammatory cytokines such as IL-1 and TNF, and antigen receptor activation (Ghosh and Karin, 2002). Conversely, the non-canonical pathway is activated by TNF-family cytokines; CD40 ligand (CD40L and TNFSF5), B cell activating family (BAFF and TNFSF13B) and receptor activator of NF- κ B ligand (RANKL and TNFSF11). These two pathways share a common regulatory step required for the activation of NF- κ B; the activation of an I κ B kinase (IKK) complex, which consists of catalytic kinase subunits (IKK α and/or IKK β) and the regulatory non-enzymatic scaffold protein NF- κ B essential modulator (NEMO). Upon activation, an IKK complex phosphorylates I κ B, leading to its polyubiquitination and proteasomal degradation. Unbound NF- κ B dimers are consequently liberated, enabling them to translocate to the nucleus and modulate target gene expression.

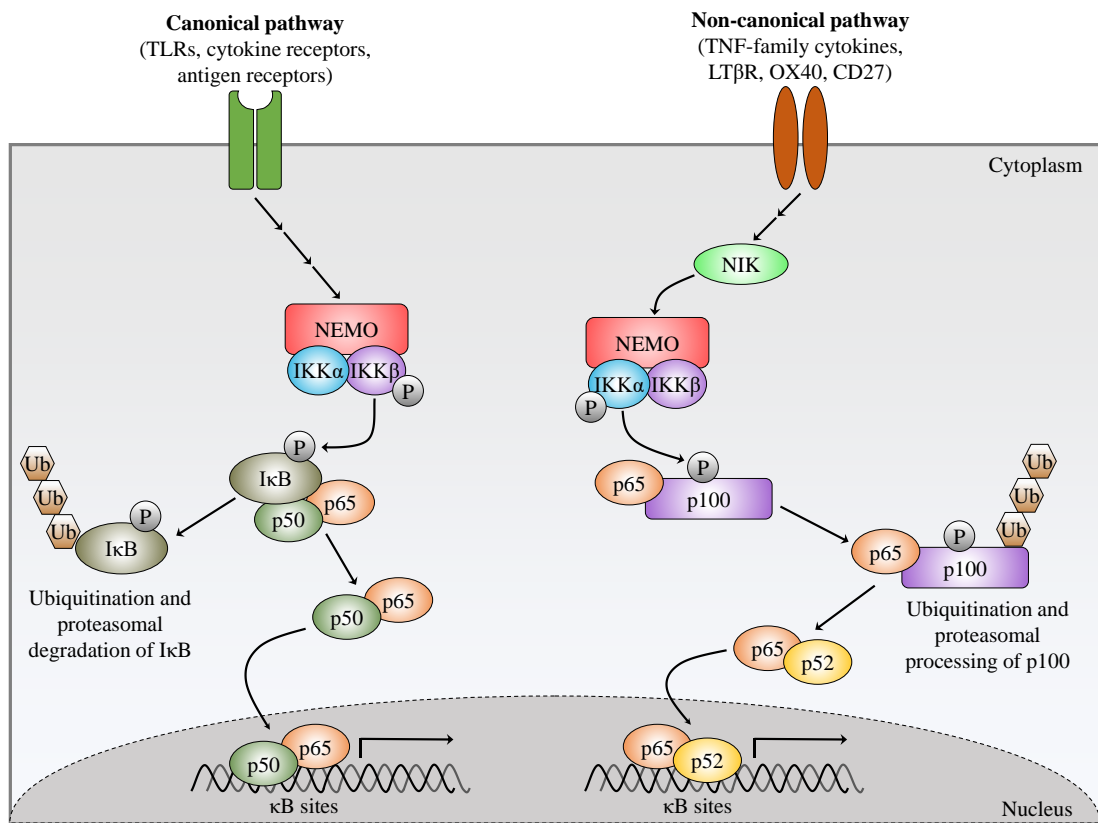


Figure 1.6 | Canonical and non-canonical pathway of NF-κB activation.

The canonical NF-κB pathway is induced by signals including antigens, TLR ligands and cytokines. This leads to the phosphorylation of the IKKβ subunit of the IKK complex (NEMO, IKKα, IKKβ). The IKK complex subsequently phosphorylates (P) IκB proteins bound to NF-κB dimers such as p50-p65 dimers, leading to polyubiquitination (Ub) and subsequent proteasomal degradation of IκB. Liberated NF-κB dimers can then translocate to the nucleus and bind specific DNA sequences (κB sites) and induce target gene expression. The non-canonical NF-κB pathway is activated by members of the TNF-like family of cytokines. This leads to NIK-mediated IKKα activation, which phosphorylates p100, initiating its proteasomal processing into p52. The p52 protein preferentially dimerizes with p65, translocates to the nucleus and induces target gene expression.

1.3.3.1.2 Regulation of NF- κ B signalling

Given the importance of NF- κ B signalling in a wide range of key physiological processes, the NF- κ B pathway is tightly regulated at multiple levels, extensively reviewed by (Oeckinghaus and Ghosh, 2009). Indeed, dysregulated NF- κ B signalling has been attributed to the development of many human diseases including inflammatory diseases such as auto-immune diseases and atherosclerosis, as well as in neurodegeneration, and cancer (Hoesel and Schmid, 2013, Tilstra et al., 2011, Vallabhapurapu and Karin, 2009).

The best characterised mechanism of NF- κ B signalling termination involves the re-synthesis of I κ B proteins, the expression of which are induced by NF- κ B signalling (Haskill et al., 1991, Sun et al., 1993, de Martin et al., 1993). Newly synthesised I κ B proteins enter the nucleus, bind to NF- κ B (removing it from DNA), and re-localise to the cytosol (Hayden and Ghosh, 2004). More recently, a number of additional mechanisms of NF- κ B signalling have been identified that directly affect active NF- κ B subunits bound to DNA. These include a wide array of post-translational modifications such as ubiquitination, phosphorylation, and acetylation (Perkins, 2006). These protein modifications alter cofactor binding or mediate the displacement and subsequent degradation of NF- κ B dimers, resulting in the downregulation of NF- κ B signalling (Kiernan et al., 2003, Saccani et al., 2004).

1.4 Soluble mediators of the inflammatory response

The release of soluble mediators following the recognition of PAMPs and/or DAMPs is a central component of the inflammatory response. PRR signalling typically culminates in the activation of transcription factors such as NF- κ B, which regulates the expression of multiple genes essential for the immune response. Tissue-resident macrophages produce a plethora of soluble mediators including cytokines, chemokines, interferons (IFNs), and lipids, which amplify the inflammatory response, participate in the activation and recruitment of effector cells, enhance phagocytosis and antigen presentation, and coordinate the adaptive response.

1.4.1 Lipid mediators

The release of lipid mediators is a characteristic hallmark of the inflammatory response, which are required for both the initial inflammatory response and the resolution of inflammation. These lipid mediators are hydrophobic, which does not permit their storage, rather they are produced *de novo* as needed. In response to pathogen recognition, innate immune cells such as macrophages produce numerous lipid mediators including members of the prostanoid family, hydroxyeicosatetraenoic acids (HETEs), leukotrienes (LTs), lipoxins (LXs), and platelet-activating factor (PAF) (Samuelsson et al., 1987, Levy et al., 2001, Flower, 2006, Godson et al., 2000).

1.4.1.1 Prostanoids

Macrophages are an important source of prostanoids, which are produced rapidly in a multi-step process following microbial recognition (Peters-Golden et al., 2005, Alvarez et al., 2010). The prostanoid family, which includes prostaglandins (PGs), prostacyclins, thromboxanes (TXs) are synthesised from lipid membrane in a multi-step process (Figure 1.7). Prostanoids are synthesised from arachidonic acid, which can either be taken up from the extracellular milieu or more commonly from arachidonic acid derived from phospholipid bilayer, which is liberated downstream of microbial recognition. Arachidonic acid is liberated from lipid membranes by the phospholipase A₂ (PLA₂) enzyme, which associates with membrane phospholipids and cleaves fatty acid, releasing arachidonic acid, γ -homolinolenic acid and eicosapentaenoic acid. Free arachidonic acid is subsequently metabolised in a multi-

step process by one of two key enzymes; cyclooxygenase (COX)-1 or COX-2. (These two proteins are encoded by two distinct genes, *Ptgs1* and *Ptgs2* respectively). Both proteins have comparable enzymatic functions, however COX-1 utilises extracellular sources of arachidonic acid, whereas COX-2 preferentially utilizes intracellular sources of arachidonic acid (Murakami et al., 1994). COX-1 and COX-2 consist of two distinct active sites that sequentially mediate the conversion of arachidonic acid to prostaglandin H₂ (PGH₂), a cyclooxygenase and a peroxidase site. Firstly, the cyclooxygenase catalyses the conversion of arachidonic acid to prostaglandin G₂ (PGG₂), after which the peroxidase site mediates the conversion of PGG₂ to PGH₂. Lastly, downstream terminal prostanoid synthases metabolise PGH₂ into members of the prostanoid family (Figure 1.7). The expression of these downstream synthases is tissue-specific, which consequently determines the profile of prostanoids produced. Resident pMφs have been reported to produce mostly arachidonic acid cyclooxygenase (COX) products including prostanoids, but not metabolites from other pathways such as the anti-inflammatory lipoxins, protectins, and resolvins (Norris et al., 2011).

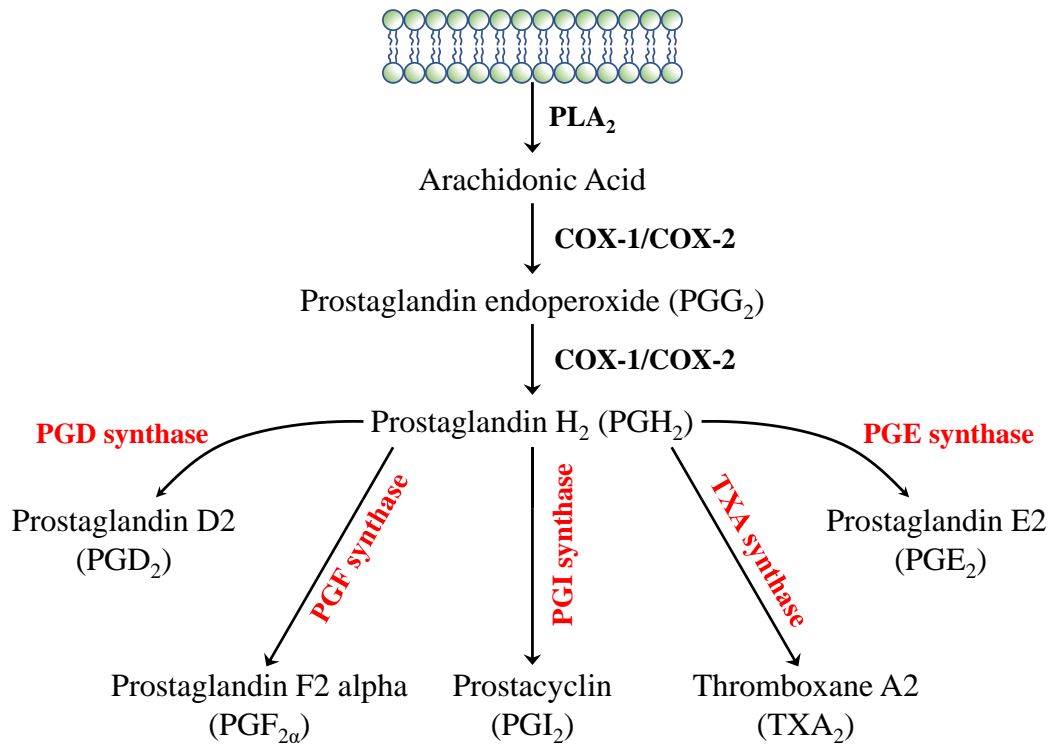


Figure 1.7 | Prostanoid synthesis pathway.

Prostanoids are synthesised from arachidonic acid, which is either liberated from the lipid membrane by PLA₂ or taken up from the extracellular milieu. COX 1/COX 2 catalyse arachidonic acid into PGG₂ and subsequently into prostaglandin H₂ (PGH₂), which is the common substrate for prostanoid synthesis. PGH₂ is processed into individual prostanoids by the action of specific terminal synthases (red text).

1.4.1.2 Prostanoid signalling

Prostanoids typically exert their biological effects locally in an autocrine or paracrine manner through binding to their cognate receptors, which are G-protein-coupled receptors (GPCRs) (Table 1.3) (Hata and Breyer, 2004). More recently, intracrine signalling has been described to be an important signalling mechanism for prostacyclin, which signals via nuclear peroxisome proliferator-activator receptors (PPARs) (Forman et al., 1997, Lim and Dey, 2002, Wise, 2003).

Table 1.3 | Prostanoid synthesis and signalling/signal transduction

Prostanoid	Key enzyme	Gene/s	Receptor/s
Prostacyclin (PGI ₂)	Prostacyclin synthase	<i>Ptgis</i>	IP PPAR γ
Thromboxane A ₂ (TXA ₂)	Thromboxane-A synthase	<i>Tbxas1</i>	TP α TP β
Prostaglandin D ₂ (PGD ₂)	Prostaglandin D ₂ synthase (PTGDS)	<i>Ptgds</i>	DP1 CRTH2
Prostaglandin E ₂ (PGE ₂)	Prostaglandin E synthase	<i>Ptges</i> <i>Ptges2</i> <i>Ptges3</i>	EP1 EP2 EP3 EP4
Prostaglandin F ₂ alpha (PGF _{2α})	Prostaglandin F ₂ alpha synthase	<i>Akr1b3</i>	FP _A FP _B

Prostanoids and prostanoid receptors in signal transduction have been extensively reviewed (Bos et al., 2004, Woodward et al., 2011).

1.4.1.3 Prostacyclin

Prostacyclin (PGI₂) is more widely recognised for its role as a potent vasodilator and inhibitor of platelet activation but its role in inflammation has become increasingly evident. The conversion of PGH₂ into PGI₂ is mediated by the enzyme prostacyclin synthase (PTGIS), which is widely expressed in vascular endothelial and smooth muscle cells, luminal epithelia, and tubal smooth muscle. Once synthesised, prostacyclin rapidly diffuses across the plasma membrane and exerts its effects through binding to its cognate receptor, the prostacyclin receptor (IP receptor). The IP receptor is a GPCR that upon activation, initiates a signalling cascade culminating in the activation of adenylyl cyclase and PLC, which subsequently increase intracellular cAMP and decreases Ca²⁺ levels. These two key secondary signalling molecules exert a wide array of effects responsible for the biological actions of PGI₂.

1.4.1.4 Prostaglandin E2 (PGE₂)

Prostaglandin E2 (PGE₂) is one of the major lipid mediators released by macrophages following TLR ligand or pro-inflammatory cytokine stimulation. It has been shown to regulate multiple aspects of inflammation and induce multiple functions in different immune cells (Phipps et al., 1991). At the early stages of inflammation, it is widely known for promoting local vasodilatation, chemoattraction, and activation of neutrophils, macrophages, and mast cells (Kalinski, 2012, Yu and Chadee, 1998). PGE₂ also stimulates the production of IL-10, whilst inhibiting the transcription of pro-inflammatory cytokines including TNF, enabling it to perturb non-specific inflammation (MacKenzie et al., 2013, Treffkorn et al., 2004). These pleiotropic effects are reflected by the existence of four different PGE₂ receptors.

1.4.2 Chemokines

During the initial inflammatory response, innate cells such as macrophages release chemokines. These small proteins, derived from ‘chemo’ (chemical) and ‘kine’ (movement) establish a chemo-attractant gradient, which enables the recruitment of (effector/immune) cells including neutrophils and monocytes and T lymphocytes (T cells) to sites of inflammation (Zlotnik and Yoshie, 2012). The recruitment is therefore important for acute effector responses and the initiation of the adaptive immune response (Ransohoff, 2009). The biological functions of chemokines are typically mediated by signalling through their cognate receptors, which are members of the GPCR family.

Chemokines can also bind to members of the atypical chemokine receptor (ACKR) family, which are structurally homologous to chemokine GPCRs. However, ACKRs fail to initiate classical signalling pathways and downstream cellular responses characteristic of GPCRs upon chemokine binding (Graham et al., 2012, Hansell et al., 2011). Furthermore, some ACKR family members have been shown to actively degrade bound chemokines. For instance, chemokines bound to ACKR2 are rapidly internalised, trafficked to endosomes where the low pH dislodges the chemokine from the receptor, and subsequently degraded in lysosomes. The unbound ACKR2 traffics back to the cell surface whereby it can re-acquire chemokines (Fra et al., 2003, Weber et al., 2004). ACKRs are therefore thought to have an important role in dampening inflammation and promoting the resolution of inflammation through the clearance of chemokines, diminishing the chemo-attractant gradient and thus influx of inflammatory cells (Griffith et al., 2014).

1.4.3 Cytokines

Cytokines are a diverse group of small, non-structural glycoproteins, which are produced by virtually all nucleated cells. They are key mediators of the inflammatory response, which amplify the initial inflammatory response, initiate the development of adaptive immunity, and promote antimicrobial effector functions (Dinarello, 2007, Arango Duque and Descoteaux, 2014, Iwasaki and Medzhitov, 2010, Turner et al., 2014). The pattern of cytokine expression varies, with some cytokines being expressed by many hematopoietic and non-hematopoietic lineages, whilst others appear to be

highly restricted to specific lineages, and differentiation or activation state. Cytokines are usually not stored as pre-formed proteins, rather their synthesis is typically induced by complement- or immunoglobulin-mediated signalling, or by microbial pathogens detected by an array of PRRs such as TLRs (Takeuchi and Akira, 2010). Ordinarily the production of cytokines is tightly regulated, whereby they are transiently produced and then repressed by multiple mechanisms. The tight regulation of cytokine production is critical for the resolution of inflammation and subsequent tissue homeostasis. Indeed, many inflammatory diseases are characterised by elevated and prolonged cytokine production.

Cytokines are predominantly short-lived and bind to specific cell surface receptors, which are differentially expressed by different cell types. Typically, they exert their effects locally in an autocrine and paracrine manner, although some including TGF- β and M-CSF are also capable of signalling in an endocrine fashion. Traditionally, cytokines were commonly classified into two broad groups, pertaining to their downstream biological effects: pro-inflammatory (e.g., IL-1 β , IL-6, IL-12, IL-18, IFN γ and TNF); and anti-inflammatory (IL-4, IL-10, IL-13, IFN α , TGF β). However, cytokines can be highly pleiotropic and as such, can exert different effects on target cells, which is dependent on several different determinants including: cell type (lineage), differentiation and/or activation states, and the presence or absence of other cytokines. With few exceptions, cytokines typically encode a signal peptide, allowing them to be synthesised, packaged, and secreted through the classical ER-Golgi secretory pathway (Stow et al., 2009).

1.4.3.1 The IL-1 family

IL-1 family members play a central role in mediating immune responses due to their diverse array of biological functions and the wide range of cell types they target. The IL-1 family of cytokines comprises 11 members: IL-1 α , IL-1 β , IL-18, IL-33, IL-36 α , IL-36 β , IL-36 γ , IL-1Ra, IL-36Ra, IL-38, and IL-37 (Table 1.4). IL-1 family members are thought to have arisen from the duplication of a common ancestral gene, based on their highly conserved gene structure, preservation of amino acid sequence, and three-dimensional structure (Dunn et al., 2001, Taylor et al., 2002b).

Typically, IL-1 family members are pro-inflammatory and mediate local and systemic inflammatory responses. Therefore, IL-1 family cytokines are tightly regulated at multiple levels by various mechanisms including receptor antagonists, decoy receptors, negative regulators, and control over the production and maturation of IL-1 family cytokines (Garlanda et al., 2013). The importance of regulation of IL-1 cytokines is exemplified in many autoimmune and inflammatory diseases to which dysregulated IL-1 signalling has been ascribed to play a critical role (Sims and Smith, 2010, Gabay et al., 2010, Dinarello, 2010, Dinarello, 2009, Dinarello et al., 2012).

Table 1.4 | IL-1 family members, their receptors, and main functions

Cytokine	Receptor	Main function
IL-1 α	IL-1R1	Agonist Pro-inflammatory
IL-1 β	IL-1R1	Agonist Pro-inflammatory
IL-18	IL-18R α	Pro-inflammatory
IL-33	ST2	Pro-inflammatory
IL-36 α	IL-36R α	Pro-inflammatory
IL-36 β	IL-36R α	Pro-inflammatory
IL-36 γ	IL-36R α	Pro-inflammatory
IL-37	IL-18R α	Anti-inflammatory – in humans only
IL-38	IL-36R	Receptor antagonist Anti-inflammatory
IL-1RA	IL-1R1	Receptor antagonist IL-1R1
IL-37RA	IL-36R	Receptor antagonist IL-36R

IL-1 family members and signalling reviewed in (Dinarello, 2009)

1.4.3.2 IL-1 Signalling

Most IL-1 family members share characteristics unlike that of other cytokines. First, IL-1 family members are predominantly synthesised as precursor proteins, with little or no biological activity, that require proteolytic processing to enable full biological activity (see 1.4.3.3 IL-1 β processing). However, some family members have been shown to engage their receptors and initiate signalling cascades in target cells, which is evident with both IL-1 α and IL-33 (Moussion et al., 2008, Dinarello, 1998). Another important feature of IL-1 family members is that except for IL-1 receptor antagonist (IL-1Ra), IL-1 family members lack a signal peptide required for conventional protein secretion, also known as leaderless proteins. However, the mechanisms governing the release of IL-1 β are poorly understood. A multitude of non-classical secretory pathways have been proposed to mediate the secretion of IL-1 β including: via exocytosis of secretory lysosomes, via exocytosis of exosomes, by shedding of plasma membrane microvesicles, and by export through plasma membrane transporters, reviewed in (Piccioli and Rubartelli, 2013, Eder, 2009).

IL-1 family members signal through a group of closely related receptors, that consists of extracellular immunoglobulin domains, a short transmembrane region, and a cytoplasmic TIR domain (Sims, 2002). IL-1 α and IL-1 β share the same cognate receptor, type 1 IL-1 receptor (IL-1R1), which is ubiquitously expressed (Dinarello, 1996). IL-1-receptor binding induces a conformational change in the extracellular domain of IL-1R1 that facilitates the recruitment of the co-receptor, IL-1R accessory protein (IL-1RAP). Formation of the trimeric complex initiates the recruitment of MyD88 and IRAK4, which perpetuate downstream signalling culminating in the activation of NF- κ B and MAPK pathways (O'Neill, 2008).

1.4.3.3 IL-1 β processing

The production and release of IL-1 β is tightly controlled through the combination of two distinct signals: transcription as well as maturation and release. The first signal (commonly referred to as the 'Priming' signal) induces the expression of the biologically inactive precursor pro-IL-1 β , which is generally mediated through NF- κ B activity induced by TLR signalling. Typically, the priming signal alone (such as LPS) fails to induce the robust processing and secretion of mature IL-1 β (Franchi et al.,

2012). The second signal ('Activation' signal) is required for the assembly of a multimeric protein complex called the inflammasome (see 1.5 Inflammasomes). The assembly of inflammasomes leads to the activation of caspase-1, which subsequently proteolytically cleaves pro-IL-1 β into the mature biologically active form (Thornberry et al., 1992). However, the requirement for two-signals for IL-1 β release seems to be species-, ligand- and cell-type-dependent specific. With few exceptions, macrophages generally require two-signals for the release of IL-1 β . Conversely, it has long been known that human blood monocytes stimulated with LPS alone secrete IL-1 β (Danis et al., 1990, Lonnemann et al., 1989, Schumann et al., 1998, Hazuda et al., 1988). Similarly, porcine whole blood and PBMCs readily secrete IL-1 β in response to LPS alone, whereas murine monocytes failed to secrete IL-1 β (Gaidt et al., 2016, Thorgersen et al., 2009). Later studies showed that human monocytes stimulated with TLR2 or TLR4 ligands alone released mature IL-1 β , which was attributed to constitutive caspase-1 activation and the release of endogenous ATP, which activates P2X₇ receptors (P2X₇R) through an autocrine loop culminating in caspase-1 activation (Netea et al., 2009, Piccini et al., 2008). Conversely, other studies indicate that IL-1 β release from human monocytes is not strictly dependent on autocrine ATP acting on P2X₇R (Ward et al., 2010, Grahames et al., 1999). Murine BMDCs have also been described to secrete mature IL-1 β in response to LPS alone (He et al., 2013, Moriwaki et al., 2015), although the mechanisms governing human monocyte and murine BMDC IL-1 β secretion in response to LPS alone are reportedly different (Gaidt et al., 2016). Thus, different cell-types have differential requirements for the processing and secretion of IL-1 β , likely reflecting their separate functions in host defence and immunity.

In addition to cell- and species-specific differential requirements for IL-1 β secretion, some ligands can act as both a 'Priming' and 'Activatory' signal and thereby induce the processing and release of mature IL-1 β . For instance, macrophages stimulated with zymosan which is recognised by both TLR2 and dectin-1 (Brown et al., 2002, Underhill et al., 1999), induce robust IL-1 β processing and secretion via a non-canonical caspase-8 inflammasome (Gringhuis et al., 2012).

1.4.3.3.1 Caspase-1 dependent processing

In general, the activation of caspase-1 is critically required for the cleavage of pro-IL-1 β and pro-IL-18. Caspase-1 is synthesized as an inactive, monomeric precursor that is predominantly activated by inflammasomes (see 1.5 Inflammasomes). In addition to the cleavage of pro-IL-1 β and pro-IL-18, caspase-1 has been demonstrated to mediate a form of cell death, termed pyroptosis, characterised by cell swelling and membrane rupture (see 1.6.4 Pyroptosis).

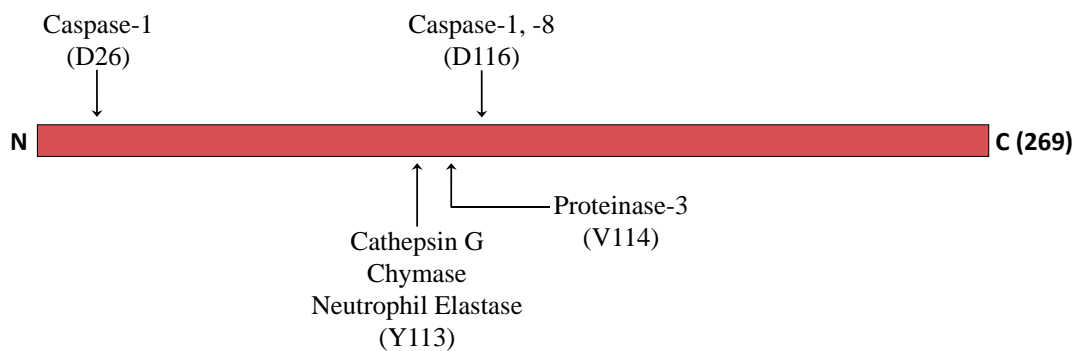


Figure 1.8 | Processing sites of pro-IL-1 β by caspase-dependent and -independent mechanisms.

Schematic representation of pro-IL-1 β protein and cleavage sites for different proteases.

1.4.3.3.2 Caspase-1-independent processing

Although caspase-1-dependent processing is the best characterised mechanism of IL-1 β processing, caspase-1-independent cleavage of IL-1 β has been reported to play a critical role in particulate-induced lung injury, host defence against pathogens, osteoarthritis, and other inflammatory diseases (Lukens et al., 2014). In support of caspase-1-independent IL-1 processing, genetic deletion of caspase-1 has been reported to not completely ablate the production of IL-1 β in several IL-1-dependent disease models (Kono et al., 2012, Lukens et al., 2012). The caspase-1-independent processing of IL-1 β has been reported to be mediated by numerous extracellular enzymes (Figure 1.8). This is particularly evident in sterile models of inflammation *in vivo*, where tissue-resident macrophages and infiltrating monocytes release pro-IL-1 β into the extracellular space upon cell death, which is subsequently cleaved by macrophage- and neutrophil-derived serine proteases including cathepsin-G, chymase,

chymotrypsin, elastase, and proteinase 3 that can cleave pro-IL-1 β to bioactive IL-1 β (Fantuzzi et al., 1997, Joosten et al., 2009).

Moreover, the proapoptotic enzyme caspase-8 has been reported to mediate IL-1 β maturation after TLR3 or TLR4 ligation (Maelfait et al., 2008). Furthermore, recombinant caspase-8 was shown to cleave pro-IL-1 β *in vitro* at the same site as caspase-1 (Asp116), suggesting a direct role for caspase-8 in the cleavage of IL-1 β (Maelfait et al., 2008). Subsequent studies have shown that a range of stimuli can activate caspase-8 to cleave IL-1 β in TLR- or TNF-primed cells including Fas death receptor ligation (Bossaller et al., 2012), and fungal recognition and binding by dectin-1, which induce the formation of a CARD9-Bcl-10-MALT1 complex (Gringhuis et al., 2012), as well as chemicals that induce ER stress, such as tunicamycin (Shenderov et al., 2014), proapoptotic chemotherapeutic compounds (for example, staurosporine and doxorubicin) (Antonopoulos et al., 2013), and histone deacetylase inhibitors (Stammli et al., 2015). Interestingly, TLR4 ligation alone can directly stimulate caspase-8 mediated IL-1 β maturation, but this mechanism is restricted to BMDCs (Moriwaki et al., 2015). Additionally, recent studies have suggested that caspase-8 can also activate the NLRP3 inflammasome. For instance, in LPS-stimulated macrophages, the chemical or genetic loss of inhibitor of apoptosis (IAP) proteins mediated ripoptosome-mediated apoptotic and necroptotic signalling leading to the activation of caspase-8 (RIP1K-RIPK3-FADD-caspase-8 signalling), which can directly cleave IL-1 β , but also induce the activation of the NLRP3 inflammasome (Lawlor et al., 2015, Vince et al., 2012, Yabal et al., 2014). The activation of the NLRP3 inflammasome by caspase-8 is suggested to be mediated by ROS activity (Vince et al., 2012), but further study is needed to fully address how RIP1K-RIP3K-FADD-caspase-8 complexes activate the NLRP3 inflammasome.

1.5 Inflammasomes

The inflammasomes are a group of multimeric protein complexes that typically consist of an inflammasome sensor molecule, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain)), and caspase-1. To date, five inflammasome sensor molecules have been demonstrated to assemble inflammasomes including members of the NLR family (NLRP1b, NLRP3 and NLRC4), as well as the receptor proteins absent in melanoma 2 (AIM2) and pyrin (Table 1.5).

Table 1.5 | Inflammasome sensor proteins and activators

Inflammasome sensor	Activators
NLRP1b	<i>Bacillus anthracis</i> lethal toxin
NLRP3	Cholesterol, Monosodium urate (MSU), Amyloid β , Extracellular ATP, Asbestos, Silica
NLRC4	Flagellin, TTSS rod component
AIM-2	Cytoplasmic dsDNA (host or microbial)
Pyrin	Inactivation of RhoA

Abbreviations: RhoA, Ras homolog gene family, member A; TTSS, type III secretion system.

Activatory stimuli of inflammasomes have been extensively reviewed by (Latz et al., 2013, Broz and Dixit, 2016).

1.5.1 NLRP3 inflammasome and its activation

1.5.1.1 Canonical NLRP3 inflammasome activation

The NLRP3 inflammasome is the best characterized inflammasome to date, which has been demonstrated to be activated (canonical-activation) by a broad spectrum of endogenous and exogenous stimuli including ATP, pore-forming toxins, and particulates such as uric acid and crystals, silica, alum adjuvant, asbestos and amyloid beta (Latz et al., 2013). The diverse array of stimuli reported to activate the inflammasome are structurally and molecularly diverse, which make it unlikely that they (all) bind directly to and activate the NLRP3 inflammasome. Therefore, it is most likely that inflammasomes monitor cellular homeostasis, which is perturbed by

activatory stimuli, and activated when they detect a specific divergence from homeostasis, such as the recognition of DAMPs. Currently, three models through which diverse stimuli converge and activate the inflammasome have been proposed: lysosomal rupture (Hornung and Latz, 2010), cytosolic K⁺ efflux (Petrilli et al., 2007), and ROS generation (Tschopp and Schroder, 2010). However, reports on their relative contributions to NLRP3 activation have often been contradictory and a single unified model of inflammasome activation has yet to be determined. More recently, on the basis of re-examination of these mechanisms, it has been proposed that the release of potassium is the downstream convergence point for *all* NLRP3 activatory stimuli (Munoz-Planillo et al., 2013, Broz and Dixit, 2016). Indeed, low potassium medium alone is sufficient to activate the NLRP3 inflammasome. However, it is currently unknown whether intracellular potassium levels modulate the activation of NLRP3 directly or whether another event correlates with low intracellular potassium concentrations is yet to be determined (Broz and Dixit, 2016). Interestingly, the activation and assembly of the apoptosome, a cytosolic molecular complex formed during apoptosis, is inhibited by physiological potassium concentrations (Cain et al., 2001). As such, potassium may regulate the threshold for NLRP3 activation, safeguarding the cell against inappropriate formation of the inflammasome from the inadvertent release and/or detection of small amount of activatory ligand (analogous to cytochrome c in the activation of the apoptosome) (Kanneganti and Lamkanfi, 2013). However, further work is needed to determine whether potassium efflux represents the secondary messenger specifically sensed by the NLRP3 inflammasome.

1.5.1.2 Mitochondrial dysfunction and inflammasome activation

Mitochondria are double-membrane-bound organelles that have critical metabolic functions including oxidative phosphorylation, fatty acid oxidation, and the Krebs cycle. In addition to their role in metabolism, mitochondria are complex regulators of cytosolic homeostasis. Perturbation of ROS, intracellular K⁺ concentration or lysosomal damage can induce mitochondrial dysfunction and apoptosis (Tschopp, 2011, Johansson et al., 2010). The role of ROS in the activation of the NLRP3 inflammasome is a topic of longstanding debate (Lawlor and Vince, 2014, Elliott and Sutterwala, 2015). Many studies implicated cytosolic ROS produced by NADPH oxidase as a critical mediator of NLRP3 activation. Furthermore, the generation of

ROS was one of the first intermediates discovered through which diverse NLRP3 activatory stimuli converge to activate the inflammasome. Since then, there have been many conflicting reports regarding the role of ROS in the activation of the NLRP3 inflammasome. Firstly, human PBMCs and mouse macrophages lacking NADPH oxidase activity (NADPH-dependent ROS production) showed normal NLRP3 inflammasome activation in response to a range of activatory stimuli (Hornung et al., 2008, van Bruggen et al., 2010, van de Veerdonk et al., 2010), suggesting that other cellular sources of ROS might be responsible for inflammasome activation. More recent studies implicated that mitochondrial ROS (mtROS) is responsible for inflammasome activation independent of NOX proteins. Dysfunctional mitochondria are thought to activate the inflammasome predominantly through the production of mtROS, and the release of mitochondrial DNA (mtDNA) and cardiolipin (Nakahira et al., 2011, Zhou et al., 2011, Iyer et al., 2013). Furthermore, oxidised mtDNA released into the cytosol during apoptosis was shown to directly bind to and activate the NLRP3 inflammasome (Shimada et al., 2012). However, the release of mtDNA can occur downstream of NLRP3 activation and warrants further investigation (Nakahira et al., 2011). Interestingly, in resting cells NLRP3 is associated with the ER but upon activation associates with mitochondria, dependent on the mitochondria anti-viral signalling protein (MAVS) (Subramanian et al., 2013, Zhou et al., 2011). However, the role of MAVS in inflammasome activation remains controversial. One report suggested a requirement for MAVS for NLRP3 activation in response to soluble stimuli (ATP, nigericin) but not particulates (alum, MSU) (Subramanian et al., 2013). However, several other reports demonstrated that MAVS was required for NLRP3 activation induced by RNA viruses (which directly engage MAVS), but expendable for non-viral stimuli including ATP and nigericin (Franchi et al., 2014, Allam et al., 2014, Park et al., 2013).

To prevent cellular damage caused by dysfunctional mitochondria, the cell has adopted several mechanisms for controlling mitochondrial quality including mitochondrial fission and fusion, the proteolytic degradation of damaged proteins, and the selective clearance of dysfunctional mitochondria by autophagy (Park et al., 2015). Indeed, impaired removal of damaged mitochondria by autophagy has been implicated in the activation of the NLRP3 inflammasome (see 1.5.1.5 Autophagy: Regulation of NLRP3 activity).

1.5.1.3 Non-canonical inflammasome activation

In addition to the wide array of stimuli that activate the NLRP3 inflammasome (canonical), a non-canonical caspase-11 dependent mechanism of NLRP3 activation has been described (Kayagaki et al., 2011). Caspase-11 predominantly detects intracellular Gram-negative bacteria in the cytosol, but also vacuolar bacteria under delayed kinetics (Aachoui et al., 2013). Thus, caspase-11 is thought to mediate protection against bacteria that escape the vacuole. More recent studies have indicated that intracellular LPS is specifically detected by caspase-11 (Hagar et al., 2013). Furthermore, caspase-11 was shown to directly bind LPS via its CARD domain, which resulted in oligomerization and activation (Shi et al., 2014). Importantly, the caspase-11 pathway is not responsive to intracellular LPS alone unless macrophages have been primed, which is necessary for the expression of caspase-11 and components of the non-canonical inflammasome (Hagar et al., 2013). The detection of Gram-negative bacteria activates the TLR4 MyD88- and TRIF-pathways, culminating in the activation of NF- κ B and subsequent transcription of IL-1 β , IL-18, and NLRP3 as well as IRF3 and IRF7 genes. IRF3-IRF7 complexes induce type I IFN signalling pathways, activating the JAK/STAT pathway and consequent the transcription of caspase-11 (Schauvliege et al., 2002, Broz et al., 2012, Rathinam et al., 2012b, Aachoui et al., 2013). In the second step, intracellular LPS binding to caspase-11 and/or unidentified scaffold proteins or receptors induce caspase-11 activation (Pellegrini et al., 2017). Once activated, caspase-11 induces pyroptosis through the cleavage of gasdermin D, as well as the activation of the NLRP3 inflammasome, culminating in the caspase-1-dependent cleavage of pro-IL-1 β and the release of mature IL-1 β (Kayagaki et al., 2015). Caspase-11 is thought to activate the NLRP3 inflammasome indirectly, as a result of K⁺ efflux through membrane pore induced by gasdermin D (Shi et al., 2015, Yang et al., 2015b).

1.5.1.4 Negative regulation of NLRP3 inflammasome

The NLRP3 inflammasome serves as a vital component to host cell defence against a number of microbial pathogens. However, emerging studies have implicated the aberrant activation of NLRP3 in a wide range of diseases, including inherited diseases (cryopyrin-associated periodic syndrome), as well as complex diseases such as multiple sclerosis, type 2 diabetes, Alzheimer's disease, atherosclerosis, obesity and gout (Wen et al., 2012, Masters et al., 2009). Accordingly, tight regulation of the

NLRP3 inflammasome at the transcriptional and post-translational levels is paramount to prevent the onset and progression of these various inflammatory and metabolic diseases, reviewed by (Rathinam et al., 2012a). Proteins composed of either a solitary CARD (CARD-only proteins; COPs) or a single pyrin domain (PYD-only proteins; POPs) have been reported to function as dominant negative molecules of inflammasome activation (Latz et al., 2013, Le and Harton, 2013). Accordingly, the expression of several COPS and POPs is induced during inflammation, suggesting that these molecules form part of the negative feedback loops that regulate the intensity and duration of the inflammatory response (Humke et al., 2000). The COP family includes COP/Pseudo-ICE (CARD16), INCA (CARD17), and ICEBERG (CARD18), caspase-12, and Nod2-S, each with varying degrees of similarity to the CARD of caspase-1. COP family members interact with the caspase-1 CARD, sequestering it from associating with ASC and thereby preventing IL-1 β and IL-18 release (Saleh et al., 2006, Druilhe et al., 2001, Lamkanfi et al., 2004). The POP family includes POP1 and POP2, which mediate their inhibitory effect on inflammasomes by interfering with the PYD-PYD interaction between NLRPs and the adaptor protein ASC (Dorfleutner et al., 2007, Bedoya et al., 2007). However, more recent studies showed that POP1 neither inhibited ASC-dependent or NLRP3 inflammasome processing of IL-1 β (Atianand and Harton, 2011). Interestingly, except for caspase-12, members of the COP and POP family are encoded by the human but not mouse genome (Chen and Sun, 2013). The presence COPS and POPs in humans suggests that they have more complexity with which inflammasomes are regulated. Alternative splicing of ASC gives rise to variants that negatively regulate inflammasome activity by inhibiting the assembly of inflammasomes (Bryan et al., 2010). Emerging evidence indicates that the tripartite-motif (TRIM) family of proteins regulate NLRP3 activity. TRIM30 was shown to suppress ROS production in response to a variety of ligands and thereby suppressing the activation of the NLRP3 inflammasome (Hu et al., 2010). The orphan nuclear receptor small heterodimer partner (SHP) was recently shown to negatively regulate NLRP3 activation, by competitively inhibiting the binding of NLRP3 to ASC (Yang et al., 2015a). Furthermore, NLRP3 activity has been reported to be regulated at the mRNA level. In one study, MicroRNA-223 (miR-223) which binds to the 3' untranslated region (UTR) of NLRP3 mRNA, suppressed NLRP3 expression and thereby reduced NLRP3 inflammasome activity (Bauernfeind et al., 2012, Haneklaus et al., 2012). Similarly, the RNA-binding protein Tristetraprolin was recently

identified to negatively regulate NLRP3 activity by targeting elements in the NLRP3 mRNA UTR and thereby repress its expression (Haneklaus et al., 2017). Type I IFNs have been shown to down-regulate the activity of NLRP3 in a STAT1-dependent manner (Guarda et al., 2011). Later studies showed that type I IFNs suppress NLRP3 activity through the activation of inducible nitric oxide synthase (iNOS), leading to the production of NO inhibits the assembly of the NLRP3 inflammasome via thiol nitrosylation (Mishra et al., 2013, Mao et al., 2013). Finally, in addition to components of the immune system, the neurotransmitter dopamine was recently shown to negatively regulate NLRP3 activity via cAMP, which binds to NLRP3 and promotes its ubiquitination and subsequent degradation (Yan et al., 2015). Similarly, cAMP generated in response PGE₂ signalling was demonstrated to inhibit NLRP3 inflammasome activity (Mortimer et al., 2016).

1.5.1.5 Autophagy

The term ‘autophagy’ (derived from the Greek words *auto* meaning ‘self’ and *phagein* meaning ‘to eat’) is a general term for the pathways by which cytoplasmic components are delivered to lysosome for degradation and has been extensively reviewed (Glick et al., 2010, Klionsky et al., 2016). To date, four different types of autophagy have been described, including microautophagy, macroautophagy, chaperone-mediated autophagy, and non-canonical autophagy. Macroautophagy (hereafter referred to as ‘autophagy’) is a critically important homeostatic mechanism for the lysosomal degradation and recycling of intracellular constituents including macromolecules and organelles (Figure 1.9) (Shintani and Klionsky, 2004). Autophagy is highly conserved in eukaryotic organisms where it is most widely recognised for its central role in the bulk degradation of intracellular components such as long-lived proteins during nutrient deprivation (Yoshimori, 2004). However, in mammals, it is now appreciated that autophagy plays important roles in a wide array of physiological processes, including innate and adaptive immune cell responses (Deretic et al., 2013). Indeed, genetic abnormalities in various essential components of autophagy have been implicated in the development of diseases including cancer, neurodegeneration, non-alcoholic fatty liver disease, and Crohn’s disease, reviewed in (Schneider and Cuervo, 2014).

1.5.1.5.1 Autophagy: Regulation of NLRP3 activity

Given the importance of autophagy in various immune pathways, it is perhaps not so surprising that autophagic dysfunction perpetuates inflammatory responses, and microbial susceptibility and pathogenesis. For instance, the ablation or reduction of the autophagy proteins Atg5, Beclin1 and LC3B were demonstrated to have elevated NLRP3 inflammasome activation and IL-1 β secretion in response to various stimuli (Zhou et al., 2011, Nakahira et al., 2011). These findings were corroborated by an earlier study where Atg16L1-deficient macrophages stimulated with LPS alone secreted IL-1 β (Saitoh et al., 2008). Later studies further indicated that autophagy can regulate the activation of the NLRP3 inflammasome in multiple ways, from the removal of endogenous inflammasome activators such as mitochondrial-derived DAMPs, to the direct sequestering and degradation of IL-1 β and inflammasome components.

Autophagy plays a central role in the clearance of dysfunctional mitochondria, in a process called mitophagy (Figure 1.9). As such, a deficiency in autophagy results in the accumulation of dysfunctional mitochondria, which is thought to contribute to the activation of the NLRP3 inflammasome through the production of ROS and release of mtDNA. Therefore, the clearance of dysfunctional mitochondria by mitophagy is essential for preventing hyperactivation of inflammatory responses. Mitophagy is a multi-step process that is initiated by PTEN-induced kinase 1 (PINK1), a serine/threonine-protein kinase which accumulates on the outer membrane of dysfunctional mitochondria with decreased inner membrane potential (Narendra et al., 2008, Okatsu et al., 2013). PINK1 subsequently recruits the E3 ubiquitin ligase (protein) Parkin from the cytosol to mitochondria. Once activated, Parkin polyubiquitinates many cytosolic and outer membrane proteins, however, the exact mechanism by which mitophagy is induced downstream of these modifications is currently unclear and widely debated. One initial report suggested that the autophagy receptor p62 (also known as SQSTM1 or sequestosome 1), was required for Parkin-mediated mitophagy (Geisler et al., 2010). p62 is a highly-conserved ubiquitin- and LC3-binding adaptor protein that was shown to mediate the aggregation and autophagosomal clearance of mitochondria (Komatsu et al., 2012). However, later reports using p62-deficient mouse embryonic fibroblasts indicated that p62 was not essential for mitophagy (Narendra et al., 2010, Okatsu et al., 2010). Conversely, one recent study showed that myeloid-specific deletion of p62 strongly enhanced LPS-induced inflammation, resulting from a deficiency in mitophagy (Zhong et al., 2016). Thus, it is most likely that these discrepancies arise from distinct cell-type and context-specific roles of p62. Interestingly, p62 expression was shown to be induced by NF- κ B signalling, suggesting that one of the mechanisms by which NF- κ B promotes the resolution of inflammation is through the induction of p62 expression, which in turn promotes the clearance of dysfunctional mitochondria by mitophagy (Zhong et al., 2016). In addition to a role in mitophagy, p62 has been suggested to attenuate inflammasome activation by targeting inflammasome components to autophagic degradation (Shi et al., 2012). Of note, autophagy has also been reported to regulate IL-1 β secretion by targeting pro-IL-1 β for lysosomal degradation, thereby limiting the intracellular pro-IL-1 β pool for NLRP3-mediated caspase-1 cleavage (Harris et al., 2011).

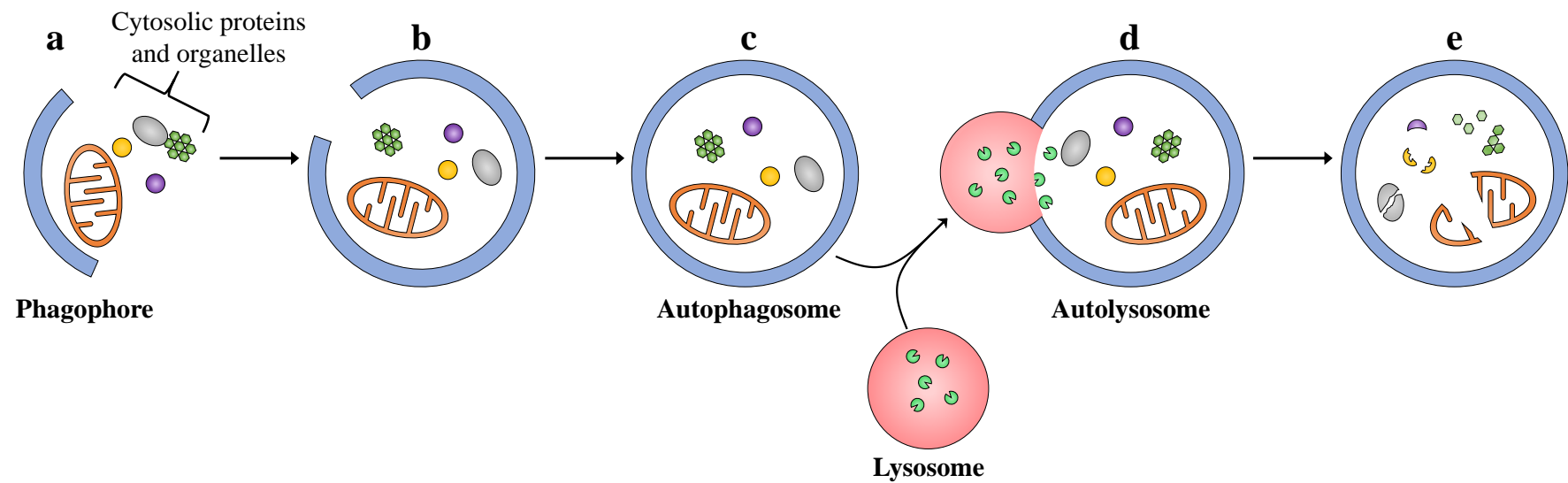


Figure 1.9 | Schematic of mammalian autophagy.

(a) Cytosolic components are sequestered by an expanding membrane, the phagophore assembly (b) The phagophore is formed upon further nucleation. (c) Phagophore membrane elongation and autophagosome completion. (d) The resulting autophagosome fuses with endocytic and lysosomal compartments, leading to the formation of autolysosomes. (e) The autophagic cargo is ultimately degraded by lysosomal hydrolases.

1.6 Cell death

In multi-cellular organisms, programmed cell death (PCD) plays a critical role in numerous biological processes including tissue remodelling during embryogenesis, the development of the immune system and during normal cell turnover. Historically, cells were thought to die by one of two modalities: apoptosis or necrosis. These cell death modalities were typically distinguished by morphological criteria such as nuclei condensation, membrane blebbing, and changes in cell size (Ziegler and Groscurth, 2004). However, with the advancement of biochemical and genetic exploration of cell death, a number of distinct PCD modalities have been identified which have been extensively reviewed by (Galluzzi et al., 2012). These advancements have led to a greater understanding of the molecular mechanisms that mediate cell death subroutines and a switch from morphological criteria to molecular definitions to distinguish different modalities (Galluzzi et al., 2012). Relevant modalities of cell death, molecular mechanisms and characteristics for this thesis are summarized in (Table 1.6).

Table 1.6 | Cell death subroutines: Molecular definitions, stimuli, and biochemical features

Cell death	Stimuli	Main biochemical features
Extrinsic apoptosis	Extracellular stress signals: FasL/CD96L, TNF, and TRAIL	Death receptor signalling: Caspase-8 (-10) activation BID cleavage and MOMP Caspase-3 (-6, -7) activation Dependence receptor signalling: PP2A activation DAPK1 activation Caspase-9 activation Caspase-3 (-6, -7) activation
Intrinsic apoptosis	Intracellular stress signals: DNA damage, oxidative stress	Caspase-dependent: Irreversible $\Delta\psi_m$ dissipation MOMP Caspase-independent: Release of IMS proteins into the cytosol Respiratory chain inhibition
Necrosis		Mitochondrial dysfunction; production of ROS, mitochondrial swelling ATP depletion Perturbed of Ca^{2+} homeostasis Physical characteristics: Lysosomal rupture Plasma membrane rupture Perinuclear clustering of organelles
Necroptosis	MLKL	RIPK1

		RIPK3
Pyroptosis	Stimuli that activate inflammasomes	Caspase-1 activation Caspase-7 activation Cleavage of GSDMD Secretion of IL-1 β and IL-18

Abbreviations: DAPK1, death-associated protein kinase-1; GSDMD, gasdermin D; IMS, mitochondrial intermembrane space; MLKL, mixed lineage kinase like.; MOMP, mitochondrial outer membrane permeabilization; PP2A, protein phosphatase 2A; TRAIL, TNF-related apoptosis-inducing ligand; $\Delta\psi_m$, mitochondrial transmembrane potential.

Modalities and molecular mechanisms of cell death subroutines are extensively reviewed by the Nomenclature Committee on Cell Death (Galluzzi et al., 2012).

1.6.1 Apoptosis

Apoptosis is a mode of PCD that occurs throughout life in multicellular organisms in which it has a critical role in development, homeostasis, and pathogenic responses. Broadly speaking, apoptosis is a form of PCD that is highly-regulated, energy-dependent and generally considered non-phlogistic (Elmore, 2007). A wide variety of physiological and pathological stimuli have been described to trigger apoptosis, for which two subroutines have been described: intrinsic and extrinsic apoptosis (Table 1.6). These stimuli induce the release of ‘find-me’ signals including nucleotides (ATP and UTP), phospholipids, and chemokines from apoptotic cells, which help to recruit phagocytes (Elliott et al., 2009). The second stage of apoptosis is the expression of ‘eat me/phagocytic’ signals on the surface of apoptotic cells. The expression of these signals is critical for their specific recognition and clearance by phagocytes (Grimsley and Ravichandran, 2003). Many phagocytic signals have been proposed including phosphatidylserine (PS), intercellular adhesion molecule-3 (ICAM3), carbohydrates, and calreticulin (Gardai et al., 2006). The exposure of phosphatidylserine (PS), an anionic phospholipid that in healthy cells predominantly resides on the cytosolic side of the plasma membrane, but is exposed to the cell surface in apoptotic cells (Fadok et al., 1992, Martin et al., 1995). In one study, PS was masked by a PS-binding protein (annexin V) which was shown to inhibit the uptake of apoptotic lymphocytes by macrophages, further implicating PS as a strong ‘eat-me’ signal candidate (Krahling et al., 1999). However, the externalisation of PS is not strictly indicative of apoptosis, as PS exposure has been reported on the surface of endothelial cells of tumour vasculature and stressed tumour cells (Ran and Thorpe, 2002, Ran et al., 2002). Furthermore, macrophages are reported to have constitutive cell surface expression of PS, which is required for the apoptotic clearance of thymocytes (Callahan et al., 2000). Numerous membrane receptors have been implicated in the recognition of apoptotic cells which directly recognise apoptotic cells including members of the T cell immunoglobulin mucin domain (TIM) protein family, stabilin 2, and brain-specific angiogenesis inhibitor 1 (BAI1). Conversely, the recognition of apoptotic cells can be mediated by soluble bridging molecules including protein S, MFGE8, and GAS6, which bind to apoptotic cells and are subsequently recognised by phagocytes (see 1.3.1.1 Humoral PRRs). However, it remains to be defined whether certain mechanisms of apoptotic recognition (membrane receptors vs soluble bridging

molecules) are only required under certain conditions (tissue development, inflammation, cell turnover), or whether these multiple mechanisms governing apoptotic cell recognition provide a degree of redundancy. Mechanisms of apoptotic recognition have recently been reviewed (Poon et al., 2014, Erwig and Henson, 2007). After phagocytosis, the apoptotic cell is processed via a phagolysosome pathway that results in the degradation of the apoptotic cell (Kinchin and Ravichandran, 2010, Kinchen et al., 2008). The final stage of apoptosis, commonly referred to as post-engulfment consequences, involves the release of anti-inflammatory cytokines and pro-resolving factors. The release of these factors typifies the predominantly immunotolerant (pro-resolving) properties of apoptosis, although under certain contexts apoptotic cells have been reported to be highly immunogenic (Casares et al., 2005, Zitvogel et al., 2004). Upon phagocytosis of apoptotic cells, macrophages release anti-inflammatory cytokines (TGF- β , IL-10) whilst concurrently inhibiting NF- κ B signalling and production of pro-inflammatory cytokines (IL-1 β , TNF, IL-12), granule enzymes, and lipids such as thromboxane B2 (Meagher et al., 1992, Fadok et al., 1998, Ipseiz et al., 2014, Voll et al., 1997). Furthermore, TLR ligands were shown to synergize with phagocytosed apoptotic cells, enhancing the production of pro-inflammatory cytokines and chemokines at early time points, but suppressing the production of pro-inflammatory cytokines and increasing the production of TGF- β at later time points compared to stimulation with TLR ligands alone (Lucas et al., 2003). This combinatorial response to apoptotic cells and TLR ligands suggests that apoptotic clearance mediates the recruitment and activation of the innate response to clear pathogens, whilst promoting later resolution of inflammation. However, if the clearance of apoptotic cells is impaired or delayed, they may begin to lose their membrane integrity and progress to secondary necrosis following a sequence of molecular events that have been extensively described (Silva, 2010, Kim et al., 2003, Vanden Berghe et al., 2010). Secondary necrotic cells release intracellular components (DAMPs) that are thought to be recognised by PRRs and provoke an inflammatory response, which is thought to contribute to a range of autoimmune and inflammatory disorders including rheumatoid arthritis, systemic lupus erythematosus, and atherosclerosis (Poon et al., 2014, Gaipal et al., 2004, Rodriguez-Manzanet et al., 2010).

1.6.2 Necrosis

Unlike apoptosis, necrosis was historically described as a form of premature cell death (non-programmed), usually triggered by external stimuli including toxins, infection, and trauma/sterile inflammation. Necrosis is characterised by a rapid loss of membrane integrity and the release of intracellular components into the extracellular space. In contrast to apoptosis, necrotic cell death *in vivo* almost invariably triggers local tissue damage and inflammation. Some of the intracellular components released by cells undergoing necrosis act as endogenous danger signals (DAMPs), that are recognised by PRRs and subsequently promote and exacerbate the inflammatory response. It has been proposed that necrosis constitutes a default cell death pathway (Golstein and Kroemer, 2007), which is supported by studies in which the inhibition of essential apoptotic machinery and autophagic proteins results in necrosis when stimulated with ligands that induce apoptosis (Shimizu et al., 2004, Degenhardt et al., 2006). Furthermore, inhibition of caspases can sensitize cells to the induction of necrosis by stimuli such as TNF; indicating that elements of the apoptotic signalling pathway might inhibit necrosis. Interestingly under these specific conditions, the spatiotemporal pattern of necrotic cell death follows that of apoptosis, indicating that necrosis simply substitutes for failed apoptosis (Golstein and Kroemer, 2007).

1.6.3 Necroptosis

Although necrosis has generally been considered a non-programmed cell death modality, in recent years, it has become increasingly evident that in some settings, necrotic cell death can be driven by defined molecular pathways, i.e., regulated necrosis. One such mechanism of regulated necrosis called ‘necroptosis’ has been described, which is characterised morphologically by organelle swelling and plasma membrane integrity loss (Pasparakis and Vandenabeele, 2015). Although TNF was reported to induce necrosis in cancer cells in the mid-1970s (Carswell et al., 1975), it was not until 25 years later than Holler and colleagues identified that receptor interacting protein kinase 1 (RIPK1 also referred to as RIP1) was the key regulatory molecule of necrosis induced by the death receptor ligands such as TNF (Holler et al., 2000). Later studies have shown that necroptosis is initiated following activation by a number of stimuli including death receptor ligands, TLRs, IFNs, and intracellular RNA and DNA sensors (Pasparakis and Vandenabeele, 2015, Thapa et al., 2013, He et al., 2011). These stimuli mediate the activation of RIPK1 and RIPK3, leading to the

formation of a pro-necrotic complex (the necrosome) through their RIP homotypic interaction motif (RHIM). The effector mixed lineage kinase like (MLKL) is recruited to the necrosome and binds to RIPK3 through its kinase-like domain. RIPK3 subsequently phosphorylates MLKL at the T357 and S358 sites, inducing a conformational change in MLKL, exposing its N-terminal death effector domain (four-helix bundle domain) (Hildebrand et al., 2014). MLKL subsequently translocates to the plasma membrane and causes its permeabilization, and thus necroptosis (Cho et al., 2009, Zhang et al., 2009, He et al., 2009, Wang et al., 2014, Dondelinger et al., 2014, Chen et al., 2013, Cai et al., 2014).

1.6.4 Pyroptosis

Pyroptosis is a lytic form of cell death that is important for host defence against pathogens but can also result in a potent and sometimes pathological inflammatory response (Jorgensen and Miao, 2015). The mechanism of IL-1 β secretion is an enduring question since the discovery that IL-1 β lacks a signal peptide, which is required for classical secretion. Pyroptosis was first characterised as an inherently pro-inflammatory form of programmed cell death (PCD) that was dependent on caspase-1 and correlated with IL-1 β secretion (Fink and Cookson, 2005). However, it was typically argued that IL-1 β release preceded cell death and/or that cell death was not intrinsically linked to the release of mature IL-1 β . Later studies showed that non-canonical inflammasome activation caused pyroptosis in a caspase-11-dependent manner (caspase-4/5 in humans) (Kayagaki et al., 2011, Shi et al., 2014). More recently, gasdermin D (GSDMD) was identified as the critical mediator of pyroptotic cell death (Shi et al., 2015, He et al., 2015, Kayagaki et al., 2015). When activated, inflammatory caspases (caspases-1, -11) cleave mouse GSDMD generating an N-terminal cleavage product (GSDMD-NT), which oligomerises in membranes to form pores and ultimately trigger pyroptotic cell death and release of IL-1 β (Liu et al., 2016). Pyroptosis is thought to be critical for the release of mature IL-1 β from the cell (Martín-Sánchez et al., 2016). Supporting this, an earlier study showed that in THP1 cells all NLRP3 stimuli tested induced necrosis, with plasma membrane permeabilization (PI uptake) coinciding with the release of IL-1 β (Cullen et al., 2015). Furthermore, viable cells stimulated with these NLRP3 activators retained mature IL-1 β , implicating membrane permeabilization as the mechanism by which mature IL-1 β is released (Cullen et al., 2015). Supporting this, single-cell imaging showed that the

activation of caspase-1 exhibits all-or-nothing (digital) activation at the single-cell level. Furthermore, IL-1 β release was only detected in dead macrophages containing active caspase-1 (Liu et al., 2014). However, this requirement is cell-type specific, as some cells release mature IL-1 β without membrane rupture, notably human monocytes and murine neutrophils (Chen et al., 2014, Gaidt et al., 2016). Interestingly, the release of mature IL-1 β from LPS-primed macrophages stimulated with different NLRP3 stimuli was inhibited when treated with punicalagin, which stabilised plasma membranes and consequently inhibited plasma membrane permeabilization (induced by GSDMD-NT) (Martín-Sánchez et al., 2016). Furthermore, both active caspase-1 and mature IL-1 β were retained inside the cell, supporting a role for punicalagin in preventing the release of IL-1 β and not by inhibiting the activation of the inflammasome and processing of IL-1 β . Strikingly, when LPS-primed neutrophils (which release IL-1 β independently of pyroptosis) were stimulated with NLRP3 stimuli in the presence of punicalagin, the release of IL-1 β was inhibited. These studies suggest an important common mechanism of IL-1 β secretion (through membrane pores), but that cell-death is differentially regulated in different cell-types.

1.7 Hypothesis

To date, limited studies have addressed how the loss of tissue-specific transcription factors effects the inflammatory responses of tissue-resident macrophage populations. As such, the work presented in this thesis aimed to identify whether Gata6 was important for the regulation of the inflammatory response and its resolution, and whether analogous factors control the development, phenotype or function of other myeloid cells.

Chapter Two

Materials and Methods

2.1 Reagents

2.1.1 Ligands

Unless otherwise stated, Ultra-pure LPS-EB from *E. coli* O111:B4 (InvivoGen) was used for the work presented in this thesis.

Ligand	Target	Source
Pam3CSK4	TLR1/2 agonist	InvivoGen
Zymosan from <i>Saccharomyces cerevisiae</i>	TLR2 and dectin-1 agonist	Invitrogen
Poly(I:C) (HMW)	TLR3 agonist	InvivoGen
Ultra-pure LPS-EB from <i>E. coli</i> O111:B4	TLR4 agonist	InvivoGen
LPS from <i>E. coli</i> O111:B4	TLR4 agonist	Sigma
Flagellin	TLR5 agonist	InvivoGen
R848 (Resiquimod)	TLR7/8 agonist	InvivoGen
CpG ODN 1826	TLR9 agonist	InvivoGen

2.1.2 Chemical compounds and their effects

Compound	Target/effect	Source
Ac-YVAD-cmk	Selective and irreversible inhibitor of caspase-1	Sigma
Adenosine	Binds to adenosine receptors, which can down-regulate pro-inflammatory cytokine and increase anti-inflammatory cytokine production	Sigma
Apyrase	Hydrolyses ATP to AMP	Sigma
ATP	Binds to P2X receptors, triggering potassium efflux	Sigma
Bafilomycin A1	Inhibits vacuolar H ⁺ ATPase, preventing the maturation of autophagic vacuoles by	Sigma

	inhibiting the fusion between autophagosomes and lysosomes	
BAPTA-AM	Cell-permeant Ca^{2+} chelator, inhibiting Ca^{2+} -dependent signalling	Life-technologies
Chloroquine	Lysosomotropic agent that prevents endosomal acidification, thereby preventing the maturation of autophagic vacuoles by inhibiting the fusion between autophagosomes and lysosomes	Tocris Bioscience
Dibutyryl-cAMP (DB)	cAMP analogue	Sigma
Enbrel® (Etanercept)	Soluble TNF receptor (blocks TNF signalling)	Kind gift from Dr Gareth Jones (Cardiff University)
Indomethacin	COX1/2 inhibitor	Cambridge Bioscience
MCC950	Selective small molecule inhibitor of NLRP3 inflammasome activation	Kind git from Dr Avril Robertson (University of Queensland)
Mito-TEMPO	Mitochondria-targeted antioxidant with alkyl and superoxide scavenging properties	Enzo Life Sciences
N-acetyl-L-cysteine (NAC)	An antioxidant which scavenges ROS	Sigma
Nigericin	Microbial toxin that acts as a potassium ionophore	Invivogen
Recombinant mouse IL-1ra	Receptor antagonist which blocks IL-1 signal transduction	Recombinant mouse IL-1ra was from R&D systems and a kind gift from Dr Selinda Orr
Rp-Adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPs)	Specific membrane-permeable inhibitor of the activation of cAMP-dependent protein kinases I and II by cAMP	Sigma
Sodium Beraprost	PGI ₂ analogue	Cambridge Bioscience
Torin2	Potent and selective mTOR inhibitor	Tocris Bioscience
Wortmannin	Specific and potent PI3-K inhibitor. Inhibits autophagic sequestration	Sigma

2.1.3 Solutions and buffers

Ammonium-Chloride-Potassium (ACK) lysis buffer (pH 7.2)

150 mM NH_4Cl

10 mM KHCO_3

0.1 mM Na_2EDTA

Annealing buffer (pH 7.5)

10 mM Tris

50 mM NaCl

1 mM EDTA

Cell-freezing medium

90% FBS

10% Dimethyl sulfoxide (DMSO)

Cell-sorting buffer

1X DPBS

0.5% (w/v) Bovine serum albumin (BSA)

2 mM EDTA

FACS permeabilization buffer

1X DPBS

0.5 % (w/v) BSA

5 mM EDTA

2 mM NaN_3

0.5% (w/v) saponin

FACS wash

1X DPBS

0.5% (w/v) BSA

5 mM EDTA

2 mM NaN_3

MACS buffer

1X DPBS

0.5% (w/v) BSA

2 mM EDTA

Mammalian lysis buffer

100 mM Tris (pH8.5)

5 mM ethylenediaminetetraacetic acid (EDTA)

0.2% (w/v) sodium dodecylsulphate (SDS)

200 mM sodium chloride (NaCl)

MUSE staining solution

1X DPBS

625 ng ml⁻¹ Propidium iodide (PI)

500 ng ml⁻¹ LDS 751

Lavage solution

1X DPBS

5 mM EDTA

Radio-Immunoprecipitation Assay (RIPA) buffer

150 mM NaCl

1% Triton X-100

0.5% sodium deoxycholate

0.1% SDS

50 mM Tris (pH 8.0)

1 mM EDTA

Running buffer (pH 8.3)

25 mM Tris

192 mM Glycine

0.1% (w/v) SDS

Stripping buffer (pH 6.7)

100 mM βME

2% (w/v) SDS

62.5 mM Tris-HCL

Transfer buffer (pH 8.3)

25 mM Tris

192 mM Glycine

20% (v/v) methanol

Tris-buffered saline (TBS) (pH 7.6)

137 mM NaCl

20 mM Tris

2.2 Animals

The animals used in this study were bred and maintained in Cardiff University's Joint Biological Services Unit in specific pathogen-free (SPF) conditions. All experiments were performed in accordance with institutional and UK home office guidelines. Unless specified otherwise, mice were sacrificed by asphyxiation by rising CO₂ concentration, followed by cervical dislocation.

2.2.1 Genotyping

Genotyping of mice was performed on genomic DNA extracted from ear punch biopsies. Ear punch biopsies (performed by JBIOS staff for genotyping purposes) were placed into 1.5 ml microcentrifuge tubes (STARLAB) into which 50 µl mammalian lysis buffer supplemented with 100 µg ml⁻¹ Proteinase K was added. The samples were incubated at 52°C for 1 hour in a Thermoshaker (Grant) with 1200 revolutions per minute (rpm). The proteinase K was inactivated by incubating samples at 72°C for 30 minutes. The tubes were left to equilibrate to room temperature, after which 400 µl UltraPure™ DNase/RNase-free water (Life Technologies) was added to each sample; which were then vortexed for 5 seconds and incubated at room temperature (RT) for 10 minutes. To distinguish *Gata6*^{F1/F1}, *Lyz2*^{+/+} ('*Gata6*-WT') from *Gata6*^{F1/F1}, *Lyz2*^{+/cre} ('*Gata6*-KO^{mye}') mice, 1 µl supernatant was used as a template in a standard PCR reaction (2.8.1), using primers specific for cre. The reaction was separated by agarose gel electrophoresis (2.8.1.1) and *Gata6*-KO^{mye} mice were identified by the presence of an amplicon.

2.2.2 Procedures

2.2.2.1 Experimental peritonitis

Experimental peritonitis was induced by a single 100 µl intraperitoneal injection of LPS O111:B4 (Sigma) in PBS (LPS dose and time-period as indicated in figure legend). Following sacrifice, total peritoneal cells were harvested by modified peritoneal lavage (see 2.3.7.2.1.1 Modified peritoneal lavage).

2.3 Mammalian cell culture

2.3.1 General cell culture

Mammalian cells were maintained in a humidified incubator at 37°C with 5% CO₂ unless otherwise stated.

2.3.2 Cryopreservation of cells

Sub-confluent cell lines were harvested and centrifuged at 350 x g for 5 minutes. The supernatant was aspirated and the cell pellet re-suspended (2-4x10⁶ cells/ml) in cell freezing medium. A 1 ml aliquot was then transferred into a cryogenic storage vial (Greiner Bio-One) which was then placed in a 'Mr. Frosty™' Freezing container (Thermo Fisher Scientific), filled with 100% isopropanol alcohol (Thermo Fisher Scientific), and subsequently stored at -80°C. The Mr Frosty Freezing container is a rate-controlled freezer, which cools at a rate of approximately 1 °C/minute.

2.3.3 Determination of cell number and viability

Unless otherwise stated, all experiments in this thesis were performed with samples that had greater than 95% cell viability, as determined by Trypan Blue or propidium iodide exclusion.

2.3.3.1 Haemocytometer

Cells were counted using a Neubauer Bright-Line™ Hemocytometer. An aliquot of harvested cells were stained with 0.4% Trypan Blue Solution and mixed thoroughly. The stained cell suspension was applied to the haemocytometer. Viable cells were distinguished from non-viable cells by the differential uptake of Trypan Blue, which is a membrane-impermeant dye that is excluded from viable cells but is taken up by non-viable cells. The total number of viable cells in each of the four large corner squares of the haemocytometer were counted under a Nikon Eclipse TS100 inverted light microscope. After counting, the concentration of viable cells in the original sample was calculated using the following formula:

$$\text{Total viable cells/ml} = \frac{\text{Total viable cells counted} \times \text{dilution factor}}{\text{Number of large squares counted}} \times 10000$$

2.3.3.2 Muse® Cell Analyser

The Muse® Cell Analyser (Merck Millipore) was primarily used to determine both the total cell number and percentage viability of cells by differential staining of two dyes; Propidium iodide (PI) and LDS 751. PI is a membrane impermeant nucleic acid-binding dye that is generally excluded from viable cells and can therefore be used to stain non-viable cells, whereas LDS 751 is a fluorescent cell-permeant nucleic acid stain that stains all nucleated cells. Cells were stained with MUSE staining solution and incubated at room temperature for 5 minutes. After incubation, the cells were analysed by the Muse® Cell Analyser to determine the total cell number and viability in the sample.

2.3.4 Recovery of cryopreserved cells

Cryopreserved cells were thawed rapidly by placing the cryogenic storage vial in a 37°C water bath until approximately 80% thawed. The vial was then decontaminated by spraying with 70% isopropanol. The cells were then removed and added to a 50 ml falcon tube containing 9 ml pre-warmed growth medium and centrifuged at 350 x g for 5 minutes. After centrifugation, the supernatant, containing DMSO, was removed and the cells re-suspended in fresh growth medium and seeded into an appropriate culture vessel. Typically, 24 hours after seeding the medium was refreshed.

2.3.5 Cell lines

2.3.5.1 Human Embryonic Kidney 293T (HEK 293T, '293T') cells

The 293T cell line is a derivative of HEK 293 cell line that contains the SV40 large T-antigen, which allows episomal replication of transfected plasmids which contain the SV40 origin of replication (DuBridge et al., 1987). 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Foetal bovine serum (FBS), 100 U ml⁻¹ penicillin, 10 µg ml⁻¹ streptomycin and 400 µM L-glutamine (Gibco®, Thermo Fisher Scientific). Cells were sub-cultured at a ratio of 1:3 to 1:8 every 2-3 days, when the cells were approximately 80% confluent. The growth medium was aspirated and the cells washed with DPBS, which was then removed. Pre-warmed Trypsin-EDTA (Thermo Fisher Scientific) was added to the tissue culture flask and incubated at 37°C for 5 minutes. After incubation, the flask was carefully gyrated to assist cell-detachment and examined under an inverted microscope to ensure

the cells had detached from the tissue culture vessel. An equal volume of complete medium was added to the flask to neutralise the Trypsin-EDTA. The cell suspension was added to a 50 ml falcon tube and centrifuged at 350 x g for 5 minutes. The supernatant was aspirated, after which complete medium was added and the cells gently re-suspended by pipetting to achieve a single-cell suspension. Appropriate aliquots of the cell suspension were then added to new tissue culture vessels.

2.3.5.2 Jurkat E6-1

Jurkat cells are an immortalised line of human T lymphocytes, established from the peripheral blood of a 14-year-old boy with acute T cell leukaemia (Schneider et al., 1977). Jurkat cells were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% FBS, 100 U ml⁻¹ penicillin, 10 µg ml⁻¹ streptomycin and 400 µM L-glutamine. The cell suspension was transferred into a sterile 50 ml falcon tube and centrifuged at 350 x g for 5 minutes. The supernatant was aspirated and the cells re-suspended in fresh growth medium and aliquoted into new tissue culture vessels.

2.3.5.3 RAW 264.7

RAW 264.7 are an immortalised murine macrophage cell line established from an ascites of a tumour induced in a male BALB/c mouse by intraperitoneal injection of Abelson murine leukaemia virus. Raw 264.7 cells were maintained in DMEM supplemented with 10% FBS, 100 U ml⁻¹ penicillin, 10 µg ml⁻¹ streptomycin and 400 µM L-glutamine. Cells were sub-cultured at a ratio of 1:5 every 3-4 days. Subcultures were prepared by scraping sub-confluent monolayers with a plastic cell scraper to detach the cells from the tissue culture vessel. The cell-suspension was transferred to a 50 ml falcon tube and the cells pelleted by centrifugation at 350 x g for 5 minutes. The supernatant was aspirated and the cells re-suspended in fresh growth medium. The cell suspension was gently pipetted to attain a single-cell suspension, which was then aliquoted into new tissue culture vessels.

2.3.5.4 Human bladder carcinoma cell line 5637 (5637)

2.3.5.4.1 5637 cell culture

Human bladder carcinoma cell line 5637 (5637) cells were maintained in RPMI 1640 supplemented with 10% FBS, 100 U ml⁻¹ penicillin, 10 µg ml⁻¹ streptomycin and 50

μ M beta-mercaptoethanol (β ME). Cells were sub-cultured as in (2.3.5.1), at a ratio of 1:6 every 2-3 days.

2.3.5.4.2 Preparation of 5637 conditioned medium (5637-CM)

5637 conditioned medium (5637-CM) was prepared by seeding 1.5×10^7 cells in 30 ml complete medium in 180 cm² tissue culture flasks, which were incubated overnight. The following day when the cells were confluent, the growth medium was aspirated, replenished with 30 ml fresh growth medium and incubated for 48 hours. After incubation, the medium was collected and centrifuged at 500 x g for 10 minutes. The cell-free supernatant was filtered through a 0.22 μ m filter (Merck Millipore), aliquoted into sterile 7 ml bijoux containers (Greiner Bio One) and stored at -20°C.

2.3.5.5 MUTZ-3

2.3.5.5.1 MUTZ-3 culture

The MUTZ-3 cell line was established from the peripheral blood of a 29-year-old man with acute myeloid leukaemia (AML). MUTZ-3 wells were maintained in complete medium (Minimum Essential Medium α (MEM α) containing ribonucleosides and deoxyribonucleosides supplemented with 20% (v/v) FBS, 100 U ml⁻¹ penicillin, 10 μ g ml⁻¹ streptomycin, and 50 μ M β ME supplemented with 10% (v/v) 5637-CM (2.3.5.4.2). MUTZ-3 cells were cultured in 12-well tissue culture plates by seeding cells at a concentration of 2×10^5 cells/ml in 2 ml medium. The medium was replenished after 3-4 days by adding 2 ml fresh medium. After another 3-4 days, the cells were transferred into a 50 ml falcon tube and pelleted by centrifugation at 350 x g for 5 minutes. The supernatant was aspirated and the cells re-suspended in fresh growth medium. The cells were counted (2.3.2) and re-seeded at 2×10^5 cells/ml in 2 ml medium in 12-well tissue culture plates.

2.3.5.5.2 Generation of MUTZ-3-derived LCs (MuLCs)

To generate MUTZ-3-derived LCs (MuLCs), 2×10^5 MUTZ-3 precursors were seeded in 12-well tissue culture plates in 2 ml complete medium (2.3.5.5.1) supplemented with 100 ng ml⁻¹ Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), 10 ng ml⁻¹ Transforming Growth Factor- β 2 (TGF- β 2) and 2.5 ng ml⁻¹ Tumor Necrosis Factor (TNF) (PeproTech) human recombinant proteins, and cultured for 10 days. The medium was refreshed on day 4 and 8 by exchanging 1 ml expended growth medium and with 1 ml fresh growth medium containing the appropriate concentration of

cytokines. On day 10, MuLCs were either harvested or stimulated to induce maturation (as outlined below).

2.3.5.5.3 MuLC maturation

To induce maturation of MuLCs, cells were stimulated with 1 $\mu\text{g ml}^{-1}$ LPS O111:B4 (Sigma) which was added directly into culture medium on day 10 for 24 hours. After incubation, immature and mature MuLCs were harvested by gentle pipetting.

2.3.6 Primary human cells

2.3.6.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and CD14⁺ Monocytes

Buffy coats (50 ml) from anonymous healthy donors were obtained from the Welsh Blood Service. The blood was pipetted to a sterile 250 ml tissue culture flask into which sterile PBS was added to bring the total volume to 180 ml. Afterwards, 15 ml Ficoll (Ficoll-Paque Plus, GE Healthcare) was added to six 50 ml falcon tubes. The diluted blood (30 ml) was carefully pipetted on top of each Ficoll layer, after which it was centrifuged for room temperature 30 minutes at 800 x g without deceleration. The Peripheral Blood Mononuclear Cells (PBMC) was carefully collected and washed three times with sterile PBS. The cells were counted after which CD14⁺ monocytes were isolated using anti-CD14⁺ MACS® MicroBeads (Miltenyi Biotec), following the protocol outlined in (2.4.2.8.1 Direct magnetic labelling).

2.3.6.2 Generation of monocyte-derived dendritic cells (MoDCs)

To generate monocyte-derived dendritic cells (MoDCs) isolated CD14⁺ monocytes were cultured in complete medium (Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% (v/v) FBS, 100 U ml⁻¹ penicillin, 10 $\mu\text{g ml}^{-1}$ streptomycin, 10 mM HEPES Buffer (Sigma Aldrich), 1% minimum essential medium non-essential amino acids (Thermofisher Scientific), 1mM sodium pyruvate (Thermofisher Scientific), and 50 μM βME). The cells were seeded in 6 well plates at a density of 2×10^6 cells per well in complete medium supplemented with 500 U ml⁻¹ GM-CSF and 500 U ml⁻¹ IL-4 (Miltenyi Biotec). The medium was replenished every two days by exchanging a third of the expended medium with fresh medium containing the appropriate concentration of cytokines. On day 7 the differentiated MoDCs could be harvested.

2.3.6.3 Generation of Monocyte-derived Langerhans Cells

To generate monocyte-derived Langerhans cells (MoLCs) isolated CD14⁺ monocytes were cultured in complete medium (RPMI 1640 supplemented with 10% FBS, 100 U ml⁻¹ penicillin, 10 µg ml⁻¹ streptomycin and 400 µM L-glutamine). The CD14⁺ monocytes were seeded at 1 x 10⁶ cells/ml in complete medium supplemented with GM-CSF (500 U ml⁻¹), IL-4 (500 U ml⁻¹) and TGF-β (10 ng ml⁻¹) (Peprotech). The expended medium was replenished on day 3 of differentiation. The differentiated MoLCs were harvested after 7 days of differentiation.

2.3.7 Primary murine cells

2.3.7.1 Bone marrow-derived macrophages (BMDMs)

2.3.7.1.1 Isolation of bone marrow (BM) precursors

After killing, the mice were sprayed with 70% isopropanol. A small incision was made to the ventral abdominal skin, anteriorly to the urethral opening. The posterior skin was then pulled down over each hind limb and over the foot. The foot was cut off just below the talocrural joint and at the acetabulofemoral joint with scissors, taking care to ensure that the femur and tibia remained intact. The soft tissue was carefully removed using scissors and forceps. Lint-free tissue was used to remove any remaining soft tissue. The bones were subsequently processed under aseptic conditions in a laminar flow hood. The bones were sterilized by placing them into a 30mm sterile plastic dish (Greiner Bio-One) containing 70% ethanol (EtOH) for 1 minute. The bones were washed twice with PBS to remove any traces of EtOH. Using sterile scissors and forceps, the epiphyses of individual femurs and tibia were removed. A 10 ml syringe with a 25G needle containing 10 ml complete medium (DMEM supplemented with 10% FBS, 100 U ml⁻¹ penicillin and 10 µg ml⁻¹ streptomycin) was inserted into the bone marrow lumen and used to flush the bones, until the bone cavity appeared white. The bone marrow exudate was collected into a sterile 50 ml falcon tube. This process was repeated for the remaining bones. The pooled cell suspension was gently pipetted up and passed through a 70 µM cell strainer into a falcon tube. The cell suspension was centrifuged at 350 x g for 5 minutes, the supernatant aspirated and the cells re-suspended in 1 ml Ammonium-Chloride-Potassium (ACK) lysis buffer and incubated for 1 minute at room temperature. After incubation, 9 ml DPBS was added to the cell suspension and mixed by gentle pipetting. The cell-suspension was then

carefully underlaid with 1 ml FBS and subsequently centrifuged at 350 x g for 5 minutes. The supernatant was aspirated and the cells washed twice with complete medium; the supernatant was aspirated, the cells re-suspended in complete medium and centrifuged at 350 x g for 5 minutes. After washing the cells were counted (see 2.3.2), after which the cells could be either frozen and stored (2.3.2) or differentiated (see 2.3.7.1.2).

2.3.7.1.2 Generation of BMDMs

Bone marrow-derived macrophages (BMDMs) were differentiated from BM precursors by seeding 1×10^7 cells onto a 145 mm bacteriological plastic dish (Greiner Bio-One) in 25 ml DMEM supplemented with 10% (v/v) FCS, 100 U/mL penicillin, 10 $\mu\text{g/mL}$ streptomycin and 20 ng mL^{-1} murine M-CSF (PeproTech). After 3 days, 25 ml complete medium was added to the cells. After another 3 days of culture, 25 ml medium was removed and replenished with 25 ml fresh complete medium. After 7 days, the adherent BMDMs could be harvested. The growth medium was aspirated and the cells washed with ice-cold DPBS, which was then removed. The cells were harvested by adding 5 ml PBS supplemented with 5 mM EDTA and 8 mg mL^{-1} of lidocaine hydrochloride monohydrate (Sigma-Aldrich) and incubated for 5 minutes at 37°C. After incubation, the cells were gently detached from the dish using a sterile cell lifter (Thermo-fisher Scientific). The cells were counted and seeded into an appropriate culture vessel for further experimentation in complete medium. The cells were counted and seeded into an appropriate tissue culture vessel for further experimentation. Typically, cells were not used for longer than 7 days after differentiation and were maintained in culture with complete medium supplemented with 20 ng mL^{-1} murine M-CSF.

2.3.7.2 Peritoneal macrophages (pM ϕ)

2.3.7.2.1 Peritoneal lavage

After sacrifice, 5 ml ice-cold lavage solution was instilled into the peritoneal cavity with a 21G-needle, taking care to ensure no organs were punctured. The needle was then either left in the peritoneal cavity, or the needle entry site plugged by drawing abdominal fat into the needle entry site. The abdomen was then gently massaged to suspend the peritoneal cells in the lavage solution. The needle was drawn to the user's left-hand side (right hand side of mouse) away from the viscera and with the bevel-

side facing inwards, the lavage solution slowly withdrawn. The mouse was also rolled so that the lavage fluid accumulated near the needle. If necessary, the needle would be withdrawn and inserted to remove any remaining lavage solution. The lavage solution was ejected into a sterile 15ml falcon tube on ice.

2.3.7.2.1.1 Modified peritoneal lavage

The production of cytokines and chemokines *in vivo* was determined in lavage supernatants following a modified peritoneal lavage protocol. After sacrifice, 1 ml ice-cold lavage solution was instilled into the peritoneal cavity with a 23G-needle and following the procedure outlined above, were aliquoted into 1.5 ml microcentrifuge tubes. The mouse was lavaged again, following the standard procedure (5 ml lavage), which was dispensed into a 15 ml falcon tube. The 1.5 ml microcentrifuge tubes were centrifuged at 350 x g for 5 minutes and the supernatant transferred to clean microcentrifuge tubes, after which they were stored at -80°C. Concurrently, the cell pellet was pooled with the second lavage for that individual mouse.

2.3.7.2.2 Phenotypic analysis of pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice

To distinguish pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice, samples were analysed by flow cytometry (see 2.4.2 General flow cytometry) for the expression of the cell surface markers F4/80 and CD73, which are differentially expressed in pMφs from *Gata6*-WT (F4/80^{high}CD73^{high}) and *Gata6*-KO^{mye} (F4/80^{low}CD73^{low}) mice (Rosas et al., 2014). Briefly, 50 µl lavage was added to V-bottom 96-well polystyrene microplate containing 50 µl FACS block and incubated on ice for 15 minutes. After incubation, FACS block containing anti-mouse PE anti-mouse F4/80 and V450 anti-mouse CD73 antibodies was added directly to each well and incubated on ice for 15 minutes. After incubation, the cells were washed twice; the cells were pelleted by centrifugation at 350 x g for 5 minutes, the supernatant aspirated, and the cells re-suspended in FACS wash. The samples were analysed by flow cytometry and pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice identified by F4/80^{high}CD73^{high} and F4/80^{low}CD73^{low} expression respectively (**Error! Reference source not found.**).

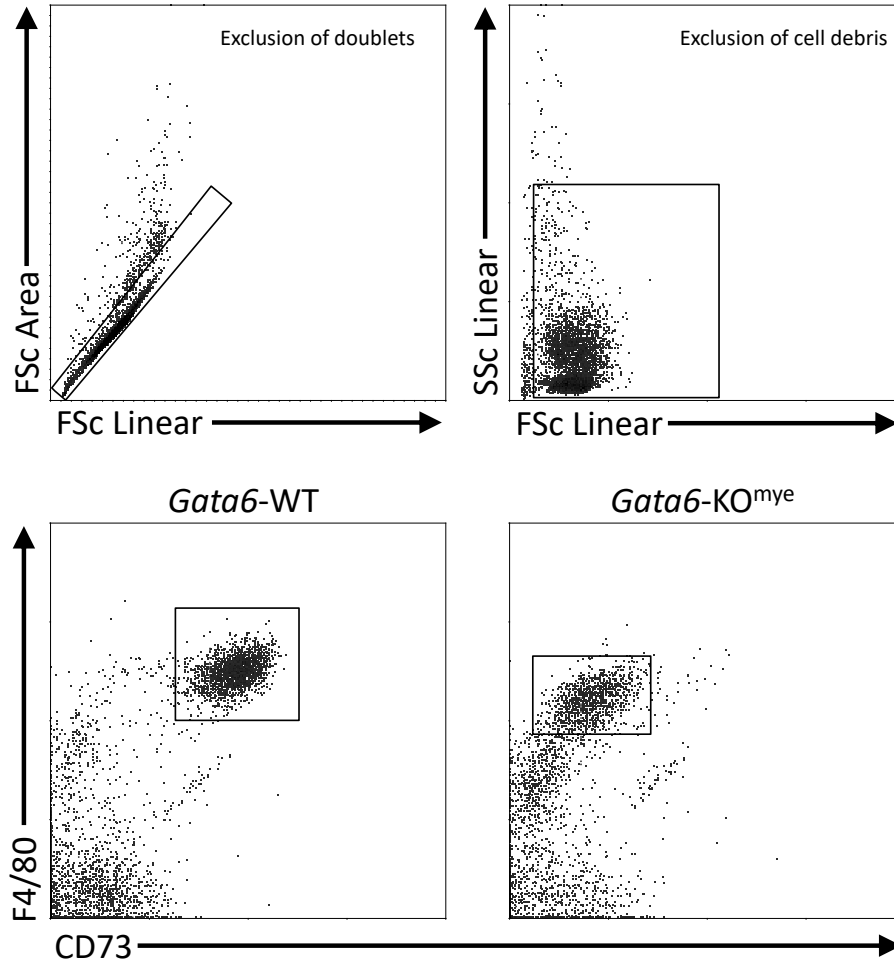


Figure 2.1 | General gating strategy and phenotypic analysis of pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice by flow cytometry.

2.3.7.2.3 General pMφ culture

Total peritoneal exudate cells (PECs) or adherence-purified peritoneal macrophages (see below) were maintained in RPMI 1640 supplemented with 10% (v/v) FBS, 100 U ml⁻¹ penicillin, 10 μg ml⁻¹ streptomycin and 400 μM L-glutamine unless stated otherwise.

2.3.7.2.4 Adherence-purification of peritoneal macrophages and *ex vivo* microbial product stimulation

The viability and total cell counts of PECs (or MACS-enriched pMφs) were determined using the Muse® Cell Analyser. The percentage of pMφs in each sample (% F4/80⁺ cells by flow cytometry) was used to ascertain the total number of pMφs in each sample. Unless otherwise stated, lavages of the same genotype were pooled and

washed three times with complete medium. After washing, 1×10^5 pMφs were seeded into each well of a 96-well tissue culture plate in 200 µl complete medium (or scaled up accordingly for larger multi-well plates at 5×10^5 cells/ml) and placed at 37°C, 5% CO₂ for 3 hours. After incubation, non-adherent cells were removed by washing the wells three times with complete medium. The adherent peritoneal exudate cells (APECs) were then either unstimulated or stimulated with various ligands in the presence or absence of chemical compounds for the indicated time-points as described in each figure.

2.3.7.2.5 *Ex vivo* microbial product stimulation of total PECs

Flow cytometric experiments were performed with total PECs, unless otherwise stated. Total PECs were seeded at 4×10^5 cells into each well of a flat-bottomed 24-well ultra-low attachment plate (Corning®) in 800 µl complete medium and treated as described in each figure legend. After the indicated time point, total PECs were harvested by first pipetting the cell suspension in each well into 15 ml falcon tubes stored on ice. The remaining cells were harvested by adding 400 µl Accumax™ solution (Sigma-Aldrich) to each well and incubated at 37°C for 10-15 minutes. After incubation, the cells were pipetted gently to suspend any remaining adherent cells. The cell suspension was subsequently pooled with the corresponding sample stored on ice.

2.3.7.2.6 Cell viability analysis

Cell viability of *ex vivo* pMφs was assessed by one of three methods, the Vybrant™ Cytotoxicity Assay, PrestoBlue™ Cell Viability, and propidium iodide (PI) flow cytometry (see 2.4.2.6 for PI viability staining).

The Vybrant™ Cytotoxicity Assay Kit (Thermo Fisher Scientific) was used to assess cell viability through monitoring the presence of a cytosolic enzyme, G6PD, which is released by dying cells into the culture medium. The cells were plated (2.5×10^4) in each well of a 96-well plate in 200 µl complete RPMI (without phenol red) and left to adhere for 3 hours. After incubation, the non-adherent cells were removed by washing the cells three times with complete medium. The cells were treated as indicated in 200 µl complete medium. In addition, separate wells reserved and lysed by adding the provided cell-lysis buffer (in triplicate) to determine the maximum G6PD release from the samples. After the indicated period, the cell supernatants were collected, centrifuged at 350 x g and the cell-free supernatants aliquoted into a fresh 96-well

plate. The supernatants were diluted in cell culture medium (1 in 10 dilution), and 50 μ l added to a 96-well black microplate (Greiner Bio-One). Cell culture medium was added in triplicate to separate wells as a no-cell control. The 2X reaction/resazurin mixture was prepared by adding 75 μ l resazurin stock (4 mM), 400 μ l reaction mixture and 9.52 ml 1X reaction buffer (scaled to accommodate a larger or smaller number of assays). The 2X reaction/resazurin mixture (50 μ l) was added to each well and mixed thoroughly. The microplate was incubated at 37 °C for 10 minutes, after which fluorescence readings were taken at 5 minute intervals for 60 minutes to determine the optimal time point. The fluorescence readings were recorded with a FLUOstar Omega plate reader (BMG LABTECH) with a 570/10 nm excitation filter and a 612/10 nm emission filter. The average values for cell culture medium alone was subtracted from all values of experimental and fully lysed cells. The percentage cytotoxicity was subsequently calculated as:

$$\text{Percent cytotoxicity} = \frac{\text{Experimental G6PD (612 nm)}}{\text{Maximum G6PD Release (612 nm)}} \times 100$$

2.3.7.2.7 PrestoBlue™ Cell Viability

PrestoBlue™ Cell Viability reagent is a resazurin-based solution which is reduced by viable cells to quantitatively measure cell viability. Cells were plated in complete RPMI (no phenol red) in 96-well clear bottom black microplates (Greiner Bio-One) and left to adhere for 3 hours. After incubation, non-adherent cells were removed by washing the cells three times with complete medium. The cells were treated as indicated after which 10 μ l PrestoBlue™ was added directly to each well and mixed by gentle pipetting. PrestoBlue™ was also added to no-cell control wells (medium alone). The cells were incubated for 60 minutes after which the fluorescence was recorded with a FLUOstar Omega plate reader (BMG LABTECH) with a 570/10 nm excitation filter and a 612/10 nm emission filter. The average fluorescence from the no-cell control wells was subtracted from the fluorescent value from the experimental cells.

2.3.7.3 Thymocytes

2.3.7.3.1 Isolation of murine Thymocytes

The mouse was sprayed with 70% isopropanol. Surgical scissors were used to make a ventral incision in the abdominal skin. The incision was extended upwards towards to

the chin of the mouse. An incision was made in the sternum and the diaphragm cut. The rib cage was cut on each side and pinned. The thymus was carefully grasped with forceps and scissors used to gently detach from appendices. The thymus was placed into DPBS on ice. Under sterile conditions, the thymus was mechanically homogenized using a sterile 2 ml syringe plunger, which was used to push the thymus through a 40 μ m cell strainer (BD Biosciences). Ice-cold DPBS was passed over the cell strainer intermittently to wash the cells through the strainer. After homogenisation, the cells were pelleted by centrifugation at 350 x g for 5 minutes. The supernatant was aspirated and re-suspended in 10 ml DPBS. The cell suspension was passed through a 40 μ m cell strainer, after which the cells were counted.

2.3.7.3.2 Generation of apoptotic thymocytes

Apoptotic thymocytes were generated by re-suspending 10^7 cells/ml in RPMI 1640 supplemented with 10% (v/v) FBS, 100 U ml⁻¹ penicillin, 10 μ g ml⁻¹ streptomycin and 1 μ M dexamethasone (Sigma-Aldrich). The cells were then incubated for 4 hours at 37 °C with 5% CO₂. After 4 hours, the thymocytes were analysed for apoptosis by flow cytometry for cleaved caspase-3, as well as cell cycle analysis with DAPI.

2.4 Flow cytometry

Table 2.1 | Antibodies used for flow cytometry

Antibody	Reactivity	Clone	Isotype	Supplier
Anti-Human				
CD1a eFluor 450	Human	HI149	Mouse IgG1, κ	eBioscience
CD207 APC	Human	10E2	Mouse IgG1, κ	Biolegend
CD80 PE	Human	2D10	Mouse IgG1, κ	Biolegend
CD83 APC	Human	HB15e	Mouse IgG1, κ	BD Biosciences
GATA3 Alexa 488	Human	L50-823	Mouse IgG1, κ	BD Biosciences
HLA-DR APC-H7	Human	L243	Mouse IgG2b, κ	BD Biosciences
Anti-Mouse				
CD11b	Mouse	5C6	Rat IgG2b	Homemade
CD11b	Mouse	M1/70	Rat IgG2b	BD Biosciences
CD73	Mouse	TY/11.8	Rat IgG1	eBioscience
Cleaved Caspase-3 (Asp175)	Mouse	D3E9	Rabbit IgG	Cell Signalling Technology
F4/80	Mouse	CI:A3-1	Rat IgG2b	AbD Serotec
F4/80	Mouse	BM8	Rat IgG2b	eBioscience
IL-1 β	Mouse	NJTEN3	Rat IgG1, κ	eBioscience
IL-6	Mouse	MP5-20F3	Rat IgG1	BD Biosciences
MHCII	Mouse	M5.114.15.2	Rat IgG2b	BioLegend
Tim4	Mouse	RMT4-54	Rat IgG2a	BioLegend
TNF	Mouse	MP6-XT22	Rat IgG1	BD Biosciences
Anti-Rat				

CD2 Alexa 488	Rat	OX-34	Mouse IgG2a	AbD Serotec
CD2 Alexa 647	Rat	OX-34	Mouse IgG2a	AbD Serotec
CD2 Biotin	Rat	OX-34	Mouse IgG2a	AbD Serotec

2.4.1 Multicolour flow cytometry

Multicolour flow cytometry was carried out using a 9-colour Cyan™ ADP analyser (Beckman Coulter). Unstained and single-colour stained experimental cells were included as controls within each multicolour experiment. Acquisition parameters were established using unstained and single-colour stained cells. Compensation matrices and data analysis were performed post-acquisition using Summit Software (v4.3) (Beckman Coulter), unless stated otherwise. Appropriate isotype or fluorescence-minus-one (FMO) controls were used as indicated.

2.4.2 General flow cytometry

Cells were aliquoted into V-bottom 96-well polystyrene microplates (Greiner bio-one) and fixed (if required) by adding ice-cold 4% formaldehyde (1X DPBS with 37% formaldehyde solution (Sigma-Aldrich)) to cells to obtain a final concentration of 1% (unless stated otherwise) and incubated on ice for 20 minutes. After fixation, cells were pelleted by centrifugation at 350 x g for 5 minutes, the supernatant removed and the cells washed with FACS wash. The cells were blocked by adding ice-cold FACS Block (FACS wash buffer supplemented with 5% serum (dependent on the host of antibody production) and 4 µg ml⁻¹ rat anti-mouse FcγR II and FcγR III receptors (2.4G2) (murine cells only)). The cells were blocked for 20-30 minutes on ice. After incubation, fluorescent antibodies were added directly to the cells in the blocking buffer, mixed by gentle pipetting and incubated on ice for 30-60 minutes. After incubation, the cells were washed twice with FACS wash; the cells were pelleted by centrifugation at 350 x g for 5 minutes, the supernatant aspirated and the cells-pellets re-suspended in FACS wash.

2.4.2.1 Intracellular staining

Intracellular staining was performed on fixed cells as described in (2.4.2), however FACS permeabilization buffer was used instead of FACS wash for the blocking and antibody staining steps.

2.4.2.2 Intracellular cytokine staining (ICS)

Intracellular cytokine (ICS) staining was performed as in (2.4.2.1), but cells were treated in the presence of GolgiPlug™ (BD Biosciences) for the duration of the experiment (maximum 12 hours). GolgiPlug™ prevents the release of cytokines containing a signal sequence (classically secreted cytokines) such as IL-6 and TNF, but not cytokines lacking signal sequences including most IL-1 family cytokines.

2.4.2.3 Intranuclear staining (INS)

Intranuclear staining was performed as in (2.4.2), however cells were instead fixed with 4% formaldehyde (final concentration). The cells were also permeabilised before blocking by re-suspending cells with methanol (stored at -20 °C) to obtain a final concentration of 90%, vortexed briefly and incubated on ice for 30 minutes. The cells were centrifuged at 350 x g, the methanol removed and the cells washed with FACS wash. The cells were pelleted by centrifugation, the supernatant was removed and the cells blocked and stained as described in (2.4.2).

2.4.2.4 Nuclear Isolation Medium (NIM)-DAPI staining

In a 1.5 ml microcentrifuge tube, 200 µl of cells (containing $1-3 \times 10^5$ cells) and 200 µl NIM-DAPI reagent (Beckman Coulter) were added and mixed by gentle pipetting. This was incubated on ice (protected from light) for 30 minutes. After incubation, 600 µl ice-cold DPBS was added and then centrifuged at 700 x g for 10 minutes. The supernatant was carefully removed, typically leaving 50 µl of supernatant to ensure that the nuclear pellet was not disturbed or lost. The nuclear pellet was then re-suspended in an appropriate volume of ice-cold DPBS and analysed on the 9-colour Cyan™ ADP analyser (2.4.1).

2.4.2.5 Mitochondrial staining

2.4.2.5.1 MitoTracker® staining

MitoTracker® dyes (Thermo Fisher Scientific) were used to stain mitochondria in live cells (Table 2.2). MitoTracker® Deep Red FM is a far red-fluorescent dye that stains mitochondria in live cells, which is dependent on mitochondrial membrane potential. Thus MitoTracker® Deep Red FM only stains live mitochondria. Conversely, MitoTracker® Green stains mitochondria independent on the mitochondrial membrane potential, thereby staining all mitochondria in a cell (mitochondrial mass/volume). MitoTracker dyes were added directly to culture medium (No Phenol Red) at a final concentration of 250 nM and incubated for 30 minutes, unless otherwise stated. After staining, the cells were washed twice with complete medium, after which they could be stained for cell surface markers and subsequently analysed by flow cytometry.

Table 2.2 | Mitochondrial dyes

Mitochondrial dye	Purpose	Aldehyde fixable
MitoTracker® Deep Red FM	Mitochondrial dye that stains live mitochondria	Yes
MitoTracker® Green FM	Mitochondrial mass/volume indicator	No
MitoSOX™ Red	Mitochondrial superoxide indicator	No
Dihydrorhodamine 123 (DHR123)	ROS indicator	No

2.4.2.5.2 Reactive oxygen species (ROS) detection

Mitochondrial ROS were detected in live cells with MitoSOX™ Red mitochondrial superoxide indicator (Thermo Fisher Scientific) or Dihydrorhodamine 123 (DHR123) (Invitrogen) for the detection of superoxide or hydrogen peroxide (H₂O₂) and hypochlorous acid respectively. Harvested cells were plated in 96-well low attachment plates, centrifuged at 350 x g for 5 minutes. The supernatant was removed and the cells

re-suspended in 100µl pre-warmed Hanks' Balanced Salt Solution (HBSS) or complete RPMI (No Phenol Red) containing 5 µM DHR123 or 5 µM MitoSOX red and incubated at 37°C for 20 minutes. After incubation, the cells were centrifuged at 350 x g for 5 minutes, the supernatant aspirated and the cells washed with pre-warmed HBSS/RPMI. The cells were stained for cell surface antigens (see 2.4.2 General flow cytometry) and subsequently analysed by flow cytometry.

2.4.2.6 Cell viability staining with propidium iodide

Cell viability was determined by PI exclusion in non-fixed cells. Cell surface antigen staining was performed as in (2.4.2), however, all steps were performed with buffers without NaN₃, which is toxic to cells. After staining of surface antigens, the cells were washed twice in FACS buffer. The cells were pelleted by centrifugation and re-suspended in FACS buffer containing 3 µM PI (2 µg ml⁻¹). The samples were analysed immediately by flow cytometry.

2.4.2.7 Fluorescence-activated cell sorting (FACS)

Harvested cells were centrifuged at 350 x g for 5 minutes to pellet the cells. The supernatant was then aspirated and the cells were re-suspended in ice-cold cell-sorting buffer and passed through a 40 µM nylon mesh cell strainer (BD Falcon) to obtain a single-cell suspension. The cell suspension was then centrifuged at 350 x g for 5 minutes to pellet the cells. The supernatant was then aspirated and cells re-suspended at a concentration of 2 x 10⁷ cells/ml in ice-cold blocking buffer (cell-sorting buffer supplemented with 5% rabbit serum (dependent on host of antibodies production) and 4 µg ml⁻¹ 2.4G2 (murine cells only). The cells were then incubated on ice for 20 minutes. After incubation, fluorescent antibodies were added directly to the cells in the blocking buffer and mixed by gentle pipetting. Unstained and single-stained cells were included for compensation set-up and voltage adjustment. The cells were then incubated on ice for 30 minutes protected from light. After incubation, the cells were centrifuged at 350 x g for 5 minutes to pellet the cells after which the supernatant was removed and the cells were washed with sorting buffer. This step was repeated. The cells were re-suspended at a concentration of 2 x 10⁷ cells/ml in ice-cold cell-sorting buffer and transferred to a sterile 5 ml polypropylene round-bottom falcon tube (BD Biosciences) and sorted on a BD FACS Aria™ III cell sorter (BD Biosciences). Cells

were sorted into a 5 ml a polypropylene falcon tube containing 500 μ l FCS stored on ice.

2.4.2.8 Magnetic-activated cell sorting (MACS)

2.4.2.8.1 Direct magnetic labelling

Cells were pelleted by centrifugation at 350 x g for 5 minutes, after which the supernatant was aspirated and the cells washed with ice-cold MACS buffer. The cell suspension was passed through a 40 μ M nylon mesh cell strainer (BD Falcon) to obtain a single-cell suspension. The cells were then centrifuged, the supernatant removed and the cells re-suspended at a concentration of 1.25×10^8 cells/ml ice-cold MACS buffer. MACS® MicroBeads specific for the cell-surface antigen were added to the cells (125 μ l/ml), mixed by gentle pipetting and incubated at 4°C for 15 minutes. After incubation, the cells were washed with ice-cold MACS buffer and centrifuged at 350 x g for 10 minutes. After incubation, the cells were washed with ice-cold MACS buffer and centrifuged at 350 x g for 10 minutes. In the meantime, a suitable MACS column was prepared by loading 500 μ l MACS buffer (MS column) onto the column. After equilibrating the column, the supernatant was aspirated from the cells and which were re-suspended in 500 μ l ice-cold MACS buffer (up to 10^8 cells). The cell suspension was applied to the MACS column and the flow-through collected into a sterile 15 ml falcon tube on ice. After the cell suspension had completely passed through the MACS column, the column was washed three times with ice-cold MACS buffer: 500 μ l MACS buffer was applied to the column and allowed to completely flow through the column into the collection tube before applying subsequent washes. After washing, the column was removed from the magnet and placed into 15 ml falcon tube on ice. The positive selection cells were eluted from the column by applying 1 ml MACS buffer and using the plunger to push the buffer through the column.

2.4.2.8.2 Indirect magnetic labelling

Cell surface antigens for which there were no available MACS® MicroBeads were first stained with a biotinylated primary antibody to the cell surface antigen, using the cell surface staining protocol as previously described (2.4.2 General flow cytometry). After staining, the cells were sorted using the protocol outlined in (2.4.2.8.1) using MACS® Anti-Biotin MicroBeads.

2.5 Metabolic analyses

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined using the XF24 Seahorse Metabolic Analyser (Seahorse Biosciences). TIM4-enriched pMφs were plated at a seeding density of 2.5×10^5 cells/well in 200 μ l complete RPMI into each well of a XF96 cell culture microplate (Seahorse Biosciences). After seeding, the cells were left to adhere for 2 hours in a 37°C incubator with 5% CO₂. After incubation, the medium was removed and 180 μ l XF Assay medium (Seahorse Biosciences) containing the indicated substrate (Table 2.3) was carefully added and placed into a 37°C incubator without CO₂ supplementation for 60 minutes. Meanwhile, the Seahorse Bioanalyser was heated to 37°C and calibrated using the sensor cartridge. After calibration was completed, the cell culture plate was added and run using the appropriate program (Table 2.4). Mitochondrial stress testing was performed by injecting Oligomycin (10 μ g ml⁻¹), FCCP (12 μ M) and Rotenone (2 μ M) and antimycin A (10 μ M).

Table 2.3 | Mitochondrial Stress Test Substrate Conditions

Substrate	Final concentration
No substrate	n/a
Glucose	25 mM
Glutamate	2 mM
Glucose + Glutamate	

Table 2.4 | Seahorse Mitochondrial Stress Test Program

Injection	Step	Duration (Minutes)	Cycles
	Mix	2	5
	Measure	3	
Oligomycin			
	Mix	2	2
	Measure	3	
FCCP			
	Mix	2	3
	Measure	3	
Rotenone/AA			
	Mix	2	2
	Measure	3	
2-DG			
	Mix	2	4
	Measure	3	

2.6 Molecular cloning

2.6.1 Restriction endonuclease digestion of lentiviral vectors

Lentiviral vectors were linearized by digestion with an appropriate restriction endonuclease (New England Biolabs). Typically, 1 µg lentiviral plasmid, 1 µl (10 units) restriction endonuclease (some restriction sites require more enzyme), 5 µl 10X NEBuffer and nuclease-free water up to a final reaction volume of 50 µl, were added to a 0.2 ml PCR tube and mixed by gentle pipetting. The reaction was incubated at 37°C for 1 hour and then 65°C for 20 minutes to inactivate the restriction enzyme. The restriction digest was assessed for successful digestion by agarose gel electrophoresis (2.8.1.1), after which the linearized plasmid could be excised from the gel with a sterile scalpel and purified (2.6.2).

2.6.2 DNA extraction/purification from agarose gels

DNA was purified from agarose gels using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). The excised agarose gel was solubilized with Buffer NTI (200 µl per 100 mg agarose) and incubated for up to 10 minutes with intermittent vortexing, until fully dissolved. The sample (up to 700 µl) was added to a NucleoSpin® Gel and PCR Clean-up Column and centrifuged at 11000 x g for 30 seconds. The flow-through was discarded and any remaining sample could be added and the centrifugation step repeated. The membrane was washed twice: 700 µl Buffer NT3 was added and centrifuged at 11000 x g for 30 seconds and the flow-through discarded. The membrane was dried by centrifugation at 11000 x g for 1 minute. The column was placed into a clean 1.5 ml microcentrifuge tube. Buffer NE or nuclease-free water (15-30 µl) was added to the column and incubated for 1 minute at room temperature. The DNA was eluted from the column by centrifugation at 11000 x g for 1 minute.

2.6.3 Small hairpin RNA (shRNA)

2.6.3.1 shRNA design

Validated shRNA sequences for target genes were obtained from Sigma, which were modified to incorporate a XhoI restriction site for the shRNA loop sequence, a U6 promoter termination sequence, and 5' and 3' 15 base pair sequences complimentary to the lentiviral vector. If validated shRNA sequences were not available, shRNA

sequences were designed. Non-overlapping sequences were selected in coding and non-coding sequences containing 19-21 nucleotides (annealing sequence) beginning with a guanosine (G) base, and preferentially a GC content between 30-50%. Candidate sequences were subjected to a nucleotide BLAST search to eliminate candidate shRNAs with unwanted targets.

2.6.3.2 shRNA oligonucleotide annealing

The complimentary forward and reverse shRNA oligonucleotides were re-suspended to 100 μ M in nuclease-free water. The complimentary shRNA constructs were annealed by adding 1 μ l of each shRNA and 23 μ l annealing buffer were added to a 0.2 ml PCR tube. The tube was heated to 95°C for 5 minutes in the PCR thermocycler, and then subsequently allowed to cool to room temperature (approximately 45 minutes) after which they could either be placed on ice or stored at -20°C for long term storage.

2.6.3.3 Cloning shRNAs into lentiviral vectors

The shRNA duplex (2.6.3.2) was cloned into the linearized lentiviral vector (2.6.1) using the In-Fusion® HD Cloning Kit (Clontech). 200 ng linearized vector, 1 μ l of 1:20 diluted annealed shRNA oligonucleotides, 2 μ l 5X In-Fusion HD Enzyme Premix and nuclease-free water to a total reaction volume of 10 μ l were added to a 0.2 ml PCR tube, mixed by gentle pipetting and incubated at 50 °C for 15 minutes. After incubation, the reaction was placed on ice or stored at -20 °C for long term storage.

2.6.4 Transformation of chemically competent cells

Transformation was performed using NEB 10-beta Competent E. coli (New England Biolabs). A vial of NEB 10-beta Competent E. coli was thawed in ice for 10 minutes, after cells were mixed by gentle pipetting and 50 μ l per transformation added to a 1.5 ml microcentrifuge tube on ice. 1 μ l ligation product/reaction (2.6.3.3) or 1 pg-100 ng purified plasmid DNA was added to the cells and mixed by flicking the tube 5 times and placed on ice for 30 minutes. After incubation, the tube was added to a heat block set at 42 °C for 30 seconds, and subsequently placed on ice for 5 minutes. SOC medium or LB medium (950 μ l) was added to the cells which were then placed in a 37 °C shaking incubator (250 rpm) for 60 minutes. Concurrently, LB agar plates supplemented with 100 μ g ml⁻¹ Ampicillin (Sigma-Aldrich) were placed at 37 °C for 60 minutes. After incubation, 10-fold serial dilutions of the cells was performed in

Super Optimal broth with Catabolite repression (SOC) medium (New England Biolabs) of which 100 µl was added onto each selection plate, distributed evenly across the plate with a sterile cell spreader (Thermo Fisher Scientific) and incubated overnight at 37 °C (12-16 hrs). After incubation, plates were sealed with Parafilm (Sigma-Aldrich) and stored at 4 °C for up to one month.

2.6.5 Screening for specific transformants by colony PCR

Colony PCR was used to determine whether individual transformants on the ampicillin-supplemented agar plates (2.6.4) contained the desired plasmid constructs. Individual colonies were picked by touching a sterile pipette tip onto a single colony, which was used to inoculate a PCR reaction mix with primers that enable the identification of plasmids with successful shRNA insert. The PCR reactions were then separated by AGE.

2.6.6 Bacterial culture/*E. coli* culture

Bacterial cultures for plasmid DNA purification (2.6.7) were grown from a single colony picked from an ampicillin-supplemented agar plate in a Stuart SI500 orbital shaking incubator at 37 °C with 225 rpm. A sterile pipette tip was used to pick a single colony, which was used to inoculate a starter culture of 5 ml lysogeny broth (LB) or 2xYT media (Sigma-Aldrich) containing 100 µg ml⁻¹ ampicillin (Sigma-Aldrich) and grown for 8 hours in a shaking incubator at 37 °C with 225 rpm. After incubation, the starter culture was diluted 1/500 to 1/1000 in 3 ml (minipreparation of plasmid DNA) or 100ml (maxipreparation of plasmid DNA) LB broth containing 100 µg ml⁻¹ ampicillin and cultured for 12-16 hours in a shaking incubator at 37 °C with 225 rpm.

2.6.7 Plasmid DNA purification

Plasmid DNA was purified from bacterial cultures (2.6.6) using the PureLink® Quick Plasmid Miniprep Kit (Thermo-Fisher Scientific) or Qiagen® Plasmid Maxi Kit (Qiagen). Plasmid DNA was eluted with UltraPure™ DNase/RNase-free water and stored at -20°C.

2.6.8 Plasmid DNA sequencing

Plasmid DNA was sequenced by sanger sequencing by Eurofins MWG Operon.

2.6.9 Preparation of bacterial glycerol stocks

Bacterial glycerol stocks were prepared by adding 0.5 ml overnight bacterial culture and 0.5 ml sterile 50% Glycerol solution into a sterile 1.5 ml microcentrifuge tube. The solution was mixed gently by inversion and subsequently placed at -80 °C.

2.6.10 Revival of bacteria from glycerol stocks

To recover bacteria from the frozen glycerol stock, a sterile pipette tip was used to scrape off frozen bacteria from the top of the storage vial. The bacteria were streaked directly into LB broth supplemented with 100 µg ml⁻¹ ampicillin (or appropriate selective antibiotic) and incubated overnight at 37 °C (12-16 hours).

2.7 Lentivirus production

2.7.1 Lentiviral plasmid co-transfection

Pseudovirus particles were produced using a second generation lentivirus plasmid packaging system (Burns et al., 1993). 293T cells were seeded in 75cm² tissue culture flasks in DMEM supplemented with 10% FBS, 100 U ml⁻¹ penicillin, 10 µg ml⁻¹ streptomycin and 400 µM L-glutamine the day before transfection, so that the cells were approximately 70-90% confluent on the day of transfection. The cells were co-transfected with lentiviral plasmids using the Effectene transfection kit (Qiagen). The media from the 293T cells was aspirated and 5 ml ice-cold DPBS added to wash the cells. The PBS was then aspirated and 12 ml complete medium added. The lentiviral plasmid (1 µg), the vesicular stomatitis virus glycoprotein (VSV-G) envelope encoding plasmid pMD.G2 (500 ng) and the packaging plasmid pCMVΔ8.91 (750 ng) were aliquoted into a 1.5 ml microcentrifuge into which condensation buffer EC (up to 300 µl total volume) was added. Enhancer (18 µl) was then added to the solution, which was vortexed briefly and incubated at room temperature for 5 minutes. Effectene (60 µl) was then added to the solution, which was vortexed for 10 seconds and incubated at room temperature for 10 minutes. The solution was then added to a 15 ml falcon tube and made up to 3 ml with complete media, which was then added drop-wise to the 293T cells, to make a total volume of 15 ml. The flask was then swirled to ensure sufficient distribution of the lentiviral plasmids across the cells. The supernatant was collected 48 hours after transfection, after which it was concentrated (2.7.2). Fresh medium was added to the cells and collected after 24 hours and concentrated.

2.7.2 Lentivirus particle concentration

Lentiviral supernatant (2.7.1) was added to a 50 ml falcon tube and centrifuged at 500 x g for 10 minutes to pellet cells and large cell debris. The cell-free supernatant was further clarified by filtering through a 0.45 µm polyethersulfone syringe filter (Sartorius). The supernatant was subsequently concentrated either by ultracentrifugation or using Lenti-X concentrator.

2.7.2.1 Ultracentrifugation

The clarified supernatant was carefully overlaid onto 3 ml 20% sucrose (Sigma-Aldrich) in a 30 ml konical™ Polypropylene tube (Beckman Coulter). The supernatant was centrifuged in an Optima™ L-100 XF Ultracentrifuge (Beckman Coulter) at 26000 rpm for 90 minutes at 4 °C. The tubes were carefully removed and under a tissue culture hood, the supernatant carefully removed. The tubes inverted on paper tissue and left for 10 minutes after which any residual supernatant was removed with tissue paper. The viral pellet was gently re-suspended in PBS or Aim V® Serum Free Medium and incubated at room temperature for 20 minutes. The virus solution was subsequently aliquoted into cryogenic storage vials and stored at -80 °C.

2.7.2.2 Lenti-X™ Concentrator

The clarified supernatant was added to a fresh 50ml falcon tube into which 4X Lenti-X concentrator (Clontech) was added to give a final concentration of 1X. The solution was mixed thoroughly by inverting the tube and incubated at 4°C for 6-24 hours. After incubation, the solution was centrifuged at 1500 x g for 45 minutes at 4°C. After centrifugation, the supernatant was removed and the pellet re-suspended in appropriate medium/PBS (1 ml per T75cm² flask), aliquoted into a cryogenic storage vial and stored at -80 °C.

2.7.3 Lentiviral titre determination

Lentiviral titres were determined by transduction of Jurkat E6-1 cells with different volumes of concentrated lentiviral supernatant (2.7.2) and determination of the expression of the eGFP or rat CD2 reporters by flow cytometry. Briefly, 2 x 10⁵ Jurkat E6-1 cells were seeded into 24-well plates in 200 µl complete RPMI. Different volumes of concentrated lentiviral particles were then added into each well, after which the total volume per well was made up to 400 µl with complete medium. The following day, 600 µl complete medium was added to each well. 48 hours after transduction, 1ml complete medium was added to each well. 72 hours post-transduction, the cells were harvested and fixed with 1% formaldehyde for 30 minutes on ice. After fixation, the cells were pelleted by centrifugation at 350 x g for 5 minutes. The supernatant was removed and the cells washed with ice-cold FACS wash. After washing, the cells were prepared as in (2.4.2), and analysed by flow cytometry.

2.7.4 Lentiviral particle transduction

2.7.4.1 *In vitro/ex vivo* transduction

Typically, cells grown *in vitro* were transduced with lentiviral particles in 24-well plates. BMDMs were seeded into 24-well plates in 200 μ l complete medium containing 2X M-CSF, after which lentiviral particles (suspended in AIM V® Medium) were added directly to each well (up to 200 μ l). After overnight incubation, 600 μ l complete medium containing 1 x M-CSF was added to each well. Cells were typically harvested 72 post-transduction for flow cytometric analysis, RNA extraction, or western blot analysis. For functional assays (TLR stimulation and cytokine production) the medium was aspirated, the cells washed, and fresh medium added.

2.7.4.2 *In vivo* transduction

The transduction of pM ϕ s *in vivo* was achieved through intra-peritoneal (i.p.) injection (up to 300 μ l volume) of lentiviral particles into mice. Unless stated otherwise, mice were sacrificed seven days after i.p. injection and the cells harvested by peritoneal lavage.

2.7.4.3 Spinoculation

The lentiviral transduction of cells *in vitro* has been reported to be substantially enhanced by centrifugal infection ('spinoculation'), which is thought to enhance cellular susceptibility by increasing viral deposition on target cell surfaces (O'Doherty et al., 2000). Therefore, cell lines that were more difficult to transduce with lentiviral particles were spinoculated. Cells (2×10^5) were aliquoted into each well of a U-bottom 96-well plate, centrifuged at 350 x g for 5 minutes and the supernatant aspirated. The cell pellets were subsequently re-suspended in complete medium and concentrated viral supernatant (up to 100 μ l total volume) was added to each well. The plate was wrapped with saran wrap and centrifuged at 1200 x g for 2 hours at 25°C. After centrifugation, the cells were re-suspended and transferred to 24-well plates and 300 μ l complete medium added to each well. The cell culture medium was refreshed as in (2.7.4.1).

2.8 Polymerase chain reaction (PCR)

2.8.1 Standard PCR

The PCR reactions were carried out using the KAPA2G Fast PCR MasterMix (Kapa Biosystems) in a total reaction volume of 25 µl. The PCR reaction mixture (Table 2.5) was added into 0.2 ml PCR tubes (Starlab/STARLAB) (after which the template DNA was added) and the PCR reactions performed in a Mastercycler® nexus gradient (Eppendorf) using the program shown in (Table 2.6), unless stated otherwise.

Table 2.5 | PCR reaction components per 25 µl reaction volume

Component	Volume (µl)	Final concentration
2X KAPA2G Fast Mastermix	10	1X (0.2 mM each dNTP, 0.5U DNA polymerase, 1.5 mM MgCl ₂)
Forward primer (100µM)	0.125	0.5 µM
Reverse primer (100µM)	0.125	0.5 µM
Template DNA	As required	<100 ng
Nuclease-free H ₂ O	Up to 25	—

Table 2.6 | PCR thermocycling conditions

Step	Temperature (°C)	Duration	Cycles
Initial denaturation	95	2 minutes	—
Denaturation	95	15 seconds	35
Annealing	60	15 seconds	
Extension	72	15 seconds/Kb	
Final extension	72	10 minutes	—

2.8.1.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate and visualise DNA fragments, including PCR products and plasmid DNA was digested with restriction enzymes (2.6.1). An appropriate percentage/size agarose gel (Table 2.7) was prepared by dissolving agarose powder (Thermo Fisher Scientific) in 1X Tris-acetate-EDTA (TAE) buffer (Invitrogen). The agarose powder was then dissolved by intermittent heating and swirling until the powder had fully dissolved. The solution was then allowed to cool to approximately 60 °C after which 0.5 µg ml⁻¹ Ethidium Bromide (Thermo Fisher Scientific) was added and mixed by gently swirling. The solution was then poured into a gel casting tray (EmbiTech), after which an appropriate comb was placed into the gel solution and allowed to cool until set. After the gel had solidified, the comb was removed and the gel was placed into a gel tank (EmbiTech) and submerged in 1X TAE buffer containing Ethidium Bromide. Samples were loaded onto the gel along with an appropriate DNA ladder (Promega). The gel was run at a 100 V and running dyes (xylene cyanol FF, bromophenol blue and orange G) contained within the DNA ladder were used to track migration during electrophoresis. After the products had been separated sufficiently, the gel was removed and visualized using the Bio-Rad Fluor-S MultiImager and analysed using Quantity One software (Bio-rad).

Table 2.7 .| Percentage agarose gel for resolution of DNA

Percentage Agarose gel (w/v)	Product size (Kb)
0.50	1 - 30
0.75	0.8 - 12
1.0	0.5 - 10
1.25	0.4 - 7
1.5	0.2 - 3
2.0	0.05 - 2

2.8.2 Quantitative real-time PCR (qPCR)

2.8.2.1 qPCR primer design

Oligonucleotide primers for qPCR were designed using the NCBI Primer-BLAST suite, with criteria optimised for qPCR as outlined in the MIQE guidelines (Bustin et al., 2009). Unless stated otherwise, primer pairs for qPCR were designed to: yield a PCR amplicon of between 70-150 bp and be exon spanning or separated by an intron of at least 1kb to minimise amplification from genomic DNA contamination giving rise to false positives.

2.8.2.2 qPCR primers

Table 2.8 |. qPCR primers

Gene	Forward	Reverse
<i>Mus musculus</i>		
<i>Casp1</i>	CACAGCTCTGGAGATGGTGA	CTTTCAAGCTTGGGCACTTC
<i>Il1a</i>	CGCTTGAGTCGGCAAAGAA ATC	CAGAGAGAGATGGTCAATGG CA
<i>Il1b</i>	ATGAAGGGCTGCTTCCAAAC	ATGTGCTGCTGCGAGATTTG

<i>Il6</i>	TCCGGAGAGGAGACTTCACA	TTGCCATTGCACAACCTCTTTCT
<i>Map3k8</i>	GCCTTGGTTGGTCGACTCT	TGCTGTCAGGTGGCTCTG
<i>Nlrp3</i>	TGGGCAACAATGATCTTGGC	TTTCACCCAACCTGTAGGCTCTG
<i>Ptgir</i>	ATGTACCGCCAACAGAGACG	CCTCGGATCATGAGAGGCAG
<i>Ptgis</i>	TCCACTCAGCGGATGTTTTC	ACAGGCGGTTTTTGACACTG
<i>Ptgs1</i>	GAGATCTGGACCTGGCTTCG	GTTGGACCGCACTGTGAGTA
<i>Ptgs2</i>	AGCCAGGCAGCAAATCCTT	CAGTCCGGGTACAGTCACAC
<i>Pycard</i>	ACTGTGCTTAGAGACATGGGC	TGGTCCACAAAGTGTCTGTT
<i>Serpinb2</i>	GGCTTTATCCTTTCCGTGTG	AGGAGCATGCTGATGTTTCC
<i>Sqstm1</i>	GCCAGAGGAACAGATGGAGTC	AGCTTGGCCCTTCCGATTC
<i>Tnf</i>	TAGCCACGTCGTAGCAAAC	ACAAGGTACAACCCATCGGC
<i>Ywhaz</i>	TTGAGCAGAAGACGGAAGGT	GAAGCATTGGGGATCAAGAA
<i>Homo sapiens</i>		
<i>CCND1</i>	ATGCCAACCTCCTCAACGAC	CGCAGACCTCCAGCATCC
<i>CCR6</i>	GTCATCACATTGGTGAGCTGG	AAGTAGAGGTGACTCTCAGGCA

<i>CD207</i>	AGGTCCAAAGTGTGAGGTTC TG	TCACATGGGGCATCATTCCA
<i>CD40</i>	ACTGATGTTGTCTGTGGTCC C	TGATAAAGACCAGCACCAAG AGG
<i>CD86</i>	ACTAGCACAGACACACGGA TG	TCAGAGGAGCAGCACCAGAG A
<i>CDH1</i>	CGTCCTGGGCAGAGTGAATT T	ACACCATCTGTGCCCACTTT
<i>CDK6</i>	CTGCAGGGAAAGAAAAGTG CAA	CTCCTCGAAGCGAAGTCCTC
<i>CLEC5</i> A	CCCAACGGCTTCATTACCAC	TCCTTTGCAAAAGTCCCTGCT
<i>GATA3</i>	CGTCCCGCCCTACTACG	CCGTCCAGCCAGGGTAG
<i>YWHA</i> Z	CCCAGAGAAAGCCTGCTCT	CCGATGTCCACAATGTCAAGT

2.8.2.3 RNA extraction

Total RNA was extracted from cells using RNeasy Plus Mini and Micro kits (Qiagen). The growth medium from adherent cells was aspirated after which 350 µl ($\leq 5 \times 10^6$ cells) or 600 µl ($\leq 1 \times 10^7$ cells) lysis buffer (Buffer RLT supplemented with 1% (v/v) β ME) was added. Non-adherent cells were pelleted by centrifugation at 350 x g for 5 minutes, the medium completely removed and then re-suspended in lysis buffer. The cell lysate was loaded onto a QIAshredder homogenizer (Qiagen) and centrifuged at 12 000 x g for 2 minutes. The cell lysate could then be stored at -80°C, or the RNA could be extracted immediately. The lysate was transferred to a genomic DNA (gDNA) Eliminator spin column and centrifuged at 8 000 x g for 30 seconds, to remove any contaminating gDNA. An equal volume of 70% ethanol was added to the eluate and mixed by gentle pipetting. The lysate-EtOH mixture (up to 700 µl) was transferred onto an RNeasy Mini ($\leq 1 \times 10^7$ cells) or Micro ($\leq 5 \times 10^5$ cells) spin column,

centrifuged at $>8000 \times g$ for 15 seconds and the flow-through discarded. Any remaining lysate-EtOH mixture could be subsequently added to the column, followed by centrifugation and the flow-through subsequently discarded. The column was washed with buffer RW1; 700 μ l buffer RW1 was added to the column, the cap carefully closed, centrifuged at $\geq 8000 \times g$ for 15 seconds and the flow-through discarded. The column was then washed twice with buffer RPE; 500 μ l was added to the column, centrifuged at $\geq 8000 \times g$ for 15 seconds and the eluate discarded. The column was washed with 500 μ l 80% EtOH and centrifuged at $\geq 8000 \times g$ for 2 minutes. The RNeasy spin column was placed into a new 2 ml collection tube and centrifuged with the lid open at $\geq 8000 \times g$ for 5 minutes. The RNeasy spin column was transferred to an RNase/DNase free 1.5 ml microcentrifuge tube (Eppendorf). RNase/DNase free water was added to the centre of the RNeasy Mini (30-50 μ l) or Micro (14 μ l) spin column and incubated for 2 minutes at room temperature. After incubation, the column was centrifuged at $\geq 8000 \times g$ for 1 minute to elute the RNA from the column, which was subsequently stored at -80°C .

2.8.2.4 Analysis and quantification of RNA

Total RNA (2.8.2.3) concentration and purity was determined by UV absorption in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). This calculates the concentration of nucleic acids by absorption at 260 nm. The 260/280 ratio is used to assess nucleic acid purity. A ratio of ~ 1.8 and ~ 2 are generally considered pure for DNA and RNA respectively. The 260/230 ratio is used as a secondary measure of nucleic acid purity, with a ratio between 2-2.2 considered pure.

2.8.2.5 First-strand cDNA synthesis

First strand cDNA was synthesised from total RNA (2.8.2.3) using the Precision nanoScript™ 2 Reverse Transcription kit (Primer Design). Up to 2 μ g total RNA could be reverse transcribed per reaction. Total RNA, 0.5 μ l Oligo-dT primer, 0.5 μ l Random nonamer primer and nuclease-free water to make a final reaction volume of 10 μ l were added to a thin-walled 0.2 ml PCR tube (STARLAB). The samples were incubated at 65°C for 5 minutes, after which the samples were placed on ice for at least 1 minute. 5 μ l nanoScript2 4X buffer, 1 μ l 10 mM dNTP mix, 3 μ l nuclease-free water and 1 μ l nanoScript2 enzyme were added to each sample and mixed by gentle pipetting. The samples were incubated at 25°C for 5 minutes and then at 42°C for 20 minutes. The

reaction was inactivated by incubating the samples at 75 °C for 10 minutes. Nuclease-free water was added to the cDNA to make a final concentration of 5 ng μl^{-1} and subsequently stored at -20 °C.

2.8.2.6 qPCR

The qPCR reactions were performed in a ViiA™ 7 Real-Time PCR System (Applied Biosystems) using the Fast SYBR® Green technology to determine relative expression values of target genes. The qPCR reactions were performed in a 20 μl reaction volume. The qPCR reaction components (Table 2.9) were added to a MicroAmp® Fast optical 96-well reaction plate (Applied Biosystems), which was sealed with MicroAmp® Optical Adhesive Film (Thermo-Fisher Scientific) and centrifuged at 350 x g for 5 minutes. The qPCR reactions were performed in triplicate and run following a two-step protocol (see Table 2.10). No-template control reactions were included for each primer pair and post-acquisition analysis of dissociation curves was used to determine the specificity of the qPCR reactions.

Table 2.9 |. Components for qPCR reaction

Component	Volume (μl)	Final concentration
2X SYBR® green PrecisionFAST™ with Low ROX Master mix (PrimerDesign)	10	1X
Forward Primer (10 μM)	0.6	300 nM
Reverse Primer (10 μM)	0.6	300 nM
cDNA (5 ng μl^{-1})	5	25 ng
RNase/DNase free water	3.8	—

Table 2.10 |. Thermocycling conditions for qPCR reactions

Step	Temperature	Duration	Cycles
Enzyme activation	95°C	2 minutes	—
Denaturation	95°C	5 seconds	40
Annealing/extension/data acquisition	60°C	20 seconds	
Dissociation curve	95°C	15 seconds	—
	60°C	1 minute	
	95°C	15 seconds	

2.8.2.7 Analysis of qPCR data

The comparative cycle threshold (C_T) method ($\Delta\Delta C_T$) was used for the analysis of qPCR data (Livak and Schmittgen, 2001). The relative expression of the genes of interest were calculated using an appropriate reference gene (indicated in figure legends).

2.9 Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed using the BD OptEIA™ kits (BD Biosciences) unless otherwise stated. All wash steps were performed in an automated Tecan HydroFlex™ microplate washer (Tecan). 100 µl PBS containing the appropriate capture antibody was added to each well of a flat-bottom 96-well EIA/RIA plate (Corning® 96 well EIA/RIA flat-bottomed), which was sealed and incubated overnight at 4 °C. Following the overnight incubation, the wells were washed twice with DPBS containing 0.05% (v/v) Tween-20 (Sigma-Aldrich). The wells were blocked by adding 200 µl blocking solution (DPBS with 10% FBS) for 1 hour at room temperature. Meanwhile, eight two-fold dilutions of the appropriate cytokine standard, from 2000 to 15.625 pg ml⁻¹, were performed in blocking solution. After blocking, the wells were washed 3 times and subsequently incubated with the cytokine standards, in triplicate and cell culture supernatants overnight at 4 °C. Following overnight incubation, the wells were washed 5 times after which 100 µl blocking solution containing biotinylated detection antibody and streptavidin-horseradish peroxidase (HRP) were added and incubated at room temperature for 1 hour. Following incubation, the wells were washed 7 times. Meanwhile, The TMB substrate kit (BD Biosciences) were prepared: the working reagent (WR) was prepared by mixing equal parts of 3,3',5,5'-Tetramethylbenzidine (TMB) and hydrogen peroxide. 100 µl of the WR was added to each well, the plate was then sealed and incubated at room temperature for 30-60 minutes. The reaction was terminated by adding 50 µl 2 M sulphuric acid (Fisher Scientific). The optical density (O.D.) at 450 nm with λ correction 570 nm was measured with a Multiskan®Spectrum microplate reader (Thermo Labsystems). A standard curve using the O.D. for the appropriate standard was plotted against the concentration of that standard from which linear regression analysis was used to interpolate the concentration of the unknown samples.

2.10 Nitric Oxide (NO) assay

The production of nitric oxide (NO) was determined using the Greiss test to measure nitrite (NO₂⁻); which is one of two primary stable metabolites of NO. A nitrite standard reference curve was prepared by performing six two-fold serial dilutions of

sodium nitrite (Sigma-Aldrich) in triplicate from 65 μM to 1.01 μM in the appropriate matched media for the experimental test. 50 μl of each nitrite standard (in triplicate) and each sample was added to each well of a flat-bottom 96-well EIA/RIA plate (Corning® 96 well EIA/RIA flat-bottomed). 50 μl of modified Griess reagent (Sigma-Aldrich) was added to the samples and nitrite standards, and incubated at room temperature (protected from light) for 15 minutes. Following the incubation, the spectrophotometric absorbance was measured at 540 nm with a Multiskan®Spectrum microplate reader (Thermo Labsystems).

2.11 Immunoblotting

2.11.1 Preparation of whole cell lysates

2.11.1.1 Preparation of whole cell lysates from adherent cells

The culture medium was aspirated and cells were washed with ice-cold DPBS. The DPBS was removed and cells were lysed in ice-cold RIPA buffer supplemented with 1X Halt™ Protease and Phosphatase Inhibitor Cocktail (1 ml lysis buffer per 10^7 cells), harvested using a plastic cell scraper and transferred to an ice-cold 1.5 ml microcentrifuge tube. The cell lysate was incubated on a roller at 4 °C for 30 minutes. After incubation, the lysate was centrifuged at 12000 x g for 20 minutes at 4 °C. The supernatant was carefully removed and added to a fresh pre-cooled 1.5 ml microcentrifuge tube and stored at -20 °C.

2.11.1.2 Preparation of whole cell lysates from non-adherent cells

Whole cell lysates were prepared from non-adherent cells by centrifuging the cell-suspension at 500 x g for 10 minutes. The supernatant was aspirated, the cells washed with ice-cold PBS and then centrifuged at 500 x g for 10 minutes to pellet the cells. The supernatant was removed and the cells re-suspended in lysis buffer and processed as in (2.11.1.1).

2.11.2 Preparation of cell culture supernatant/concentration

Cell-free culture supernatants were prepared by collecting the cell culture supernatants and centrifuging at 500 x g for 10 minutes. The supernatants were aspirated and placed into 1.5 ml microcentrifuge tubes, after which they could be stored at -80 °C until use. Cell-free culture supernatants were concentrated using Methanol/Chloroform precipitation. One volume of methanol and one-quarter volume of chloroform were added to the supernatant, vortexed vigorously for 20 seconds and then centrifuged at 20000 x g for 10 minutes. After centrifugation, the upper phase was aspirated, being careful not to disturb the interface. One volume of methanol was added, vortexed for 20 seconds and then centrifuged at 20000 x g for 5 minutes. The supernatant was removed and the pellets briefly dried in a heat block set at 55 °C for 10 minutes. After

drying, the pellet was re-suspended in 1 x laemmli buffer (Bio-Rad), vortexed and heated to 99 °C for 5 minutes. After heating, the sample was centrifuged at 350 x g for 1 minute to pellet insoluble components.

2.11.3 Protein Quantification/Bicinchoninic acid (BCA) protein assay

The BCA protein assay kit (Pierce) was used to determine the total protein concentration of whole cell lysate samples (2.11.1). The BCA Working Reagent (WR) was prepared by mixing 50 parts of BCA Reagent A with 1 part Reagent B. 100 µl WR was then added to each well of a flat-bottom 96-well EIA/RIA plate (Corning® 96 well EIA/RIA flat-bottomed). 5 µl of each sample (samples could be diluted in RIPA lysis buffer) and BSA standards (Seven two-fold serial dilutions of BSA from 2000 to 15.625 µg ml⁻¹) were added to each well, after which the plate was sealed and incubated at 37°C for 30-60 minutes. After incubation, the O.D. at 562 nm was measured with a Multiskan®Spectrum microplate reader (Thermo Labsystems). Protein concentrations of the unknown samples are then determined by interpolation of the BSA standard curve.

2.11.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Mini-PROTEAN® Tetra Vertical Electrophoresis System (Bio-Rad). SDS-PAGE resolving gels were made to the required percentage for resolving the protein of interest (see Table 2.11) by adding dH₂O, 30% Acrylamide/Bis Solution (37.5:1 mixture), 1.5M Tris-HCl (pH 8.8), 10% (w/v) SDS, 10% (w/v) ammonium persulfate (APS) and N,N,N',N'-Tetramethylethylenediamine (TEMED) (see Table 2.12). The solution was mixed thoroughly and pipetted into a Mini-PROTEAN® Cell 1.5 mm glass cassette (BioRad). Immediately after pouring the resolving gel, water-saturated n-butanol was used to overlay the monomer solution. The gel was left to polymerise for approximately 60 minutes, after which the overlay solution was removed and the top of the gel washed with dH₂O. The stacking gel was prepared, mixed thoroughly and added on top of the resolving gel. A 1.5 mm comb was carefully inserted to form wells and the gel left to polymerise for approximately 45 minutes.

After polymerisation, the comb was carefully removed and the wells washed with dH₂O. The SDS-PAGE gel was inserted into a cassette and placed into the Mini-PROTEAN® Tetra Vertical Electrophoresis tank (Bio-Rad). Running buffer was added to the tank until the cassette was completely submerged. The samples and ECL Full-Range Rainbow Molecular Weight Markers (Amersham) were loaded into each well. The gel was electrophoresed at 80 V until the samples had migrated to the resolving gel, after which the voltage was increased to 100 V and left until the size of interest bands had been sufficiently separated.

Table 2.11 |. Percent of acrylamide in resolving gel for separation of different molecular weight proteins

Resolving gel acrylamide percentage (%)	Protein size (kDa)
8	25-200
10	15-100
12.5	10-70
15	12-45
20	4-40

Table 2.12 |. Recipes for SDS-PAGE stacking and resolving gels

Component	Stacking Gel	Resolving Gel			
	4%	7.5%	10%	12%	15%
dH ₂ O	6ml	4.8ml	4ml	3.3ml	2.3ml
30% Acrylamide/Bis Solution (37.5:1 mixture)	1.3ml	2.5ml	3.3ml	4ml	5ml
0.5M Tris-HCl, pH 6.8	2.5ml	—	—	—	—
1.5M Tris-HCl, pH 8.8	—	2.5ml	2.5ml	2.5ml	2.5ml
10% (w/v) SDS	100μl	100μl	100μl	100μl	100μl
10% (w/v) APS	50μl	50μl	50μl	50μl	50μl
TEMED	5μl	5μl	5μl	5μl	5μl

2.11.5 Membrane transfer

Proteins that had been separated by SDS-PAGE (2.11.4) were transferred onto nitrocellulose or PVDF membranes using either a wet transfer or semi-dry transfer procedure. The gel was removed from the glass cassette and washed with transfer buffer. PVDF membranes were soaked in 100% methanol for 5 minutes, after which they were washed in distilled water for 5 minutes. After washing, the membrane was equilibrated in transfer buffer for 10 minutes.

2.11.5.1 Wet transfer

Wet transfer was performed using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The gel transfer cassette was assembled as followed from cathode to anode side: fibre pad, filter paper, gel, nitrocellulose or PDVF membrane, filter paper and fibre pad. After assembly, the cassette was placed into the transfer cell and completely submerged with transfer buffer. An ice pack was added to the transfer cell and the transfer carried out at 0.23Amps for 90 minutes.

2.11.5.2 Semi-dry transfer

Semi-dry transfer was conducted using the Trans-Blot Semi-Dry Transfer Cell (Bio-Rad). The gel sandwich was assembled as followed from the anode (bottom) to cathode (cathode top): pre-wet filter paper, membrane, gel, and pre-wet filter paper. The stainless-steel lid (cathode) and safety cover were secured in place. The blot was run at a constant voltage of 20 V for 25-40 minutes, depending on the size of the protein of interest.

2.11.6 Immunoblotting

After transfer, the membrane was washed with 1X Tris-buffered saline (TBS) for 5 minutes at room temperature on an (orbital shaker). The membranes were then blocked with blocking buffer (TBS with 0.1% (v/v) Tween® 20 (TBST) supplemented with non-fat dry milk or BSA (dependent on antibody), see Table 2.13) for 1-2 hours at room temperature. The membrane was removed and washed three times for 5 minutes each with TBST. The membrane was incubated with the primary antibody in the appropriate blocking buffer and incubated overnight at 4 °C on a roller. The following day, the membrane was washed five times for 5 minutes with TBST. The membrane was incubated with the appropriate secondary antibody in blocking buffer and incubated at room temperature for 1 hour. The membrane was washed seven times for 5 minutes with TBST. Amersham ECL Prime Western Blotting Detection Regent (GE Healthcare) was added to the membrane and incubated at room temperature (protected from light) for 5 minutes. After incubation, the membrane was removed, wrapped in plastic wrap and placed into an exposure cassette. The membrane was exposed to Super RX film (FUJIFILM) and developed using the Compact X4 imaging system (Xograph).

Table 2.13 |. Antibodies for immunoblotting

Antibody	Source	Source	Product code	Dilution factor	Blocking buffer	Staining buffer
Primary antibodies						
Anti-Mouse Ptgis	Polyclonal Rabbit IgG	Abcam	ab23668	1:2500	5% (w/v) milk	5% (w/v) milk
Anti-Mouse IL-1 β	Polyclonal Goat IgG	R&D Systems	AF-401-NA	1:500	5% (w/v) milk	0.05% (w/v) milk
Secondary antibodies						
Anti-Goat IgG-HRP	Rabbit	Santa Cruz Biotechnology	sc-2922	1:5000		0.05% (w/v) milk
Anti-Rabbit Immunoglobulins-HRP	Polyclonal Goat	Dako	P0448	1:2000		5% (w/v) milk

2.11.7 Stripping and re-probing

After film development, the membrane was washed three times for 5 minutes each with TBST. After washing, the membrane was incubated with stripping buffer (100mM β ME, 2% (w/v) SDS and 62.5mM Tris-HCL pH 6.7) and incubated at 50°C for 30 minutes with gentle agitation. The membrane was washed five times for 5 minutes each with TBST. To ensure the membrane had been sufficiently stripped, the membrane was incubated ECL Prime Western Blotting Detection Regent for 5 minutes and subsequently exposed to Super RX film and developed as in (2.11.6). If the stripping was effective, the membrane was washed for 5 minutes with TBST, after which it was suitable for immunoblotting as in (2.11.6).

2.12 Lipid extraction and analysis

Cell-free culture supernatants (1 ml) were added to glass extraction tubes into which the appropriate deuterated internal standards were spiked. 2.5 ml extraction solvent (1 M acetic acid/2-Isopropanol/Hexane (2:20:30, v/v/v) was added and vortexed thoroughly for 1 minute. 2.5 ml hexane was added and vortexed for 1 minute. The samples were centrifuged at 500 x g for 5 minutes at 4°C. After centrifugation, the upper layer was carefully removed with a glass Pasteur pipette and transferred to a clean glass vial. A further 2.5 ml hexane was added to the lower layer, vortexed thoroughly and centrifuged at 500 x g for 5 minutes at 4°C. The upper layer was removed as before and pooled with the previous layer. The samples were dried under a N₂ vacuum extraction system, after which the samples were re-suspended in 100 µl methanol. The samples could then be stored at -80°C until further analysis. Standard curves were generated from internal standards and lipid products quantified by LC/MS/MS using a 4000 Q-Trap™ LC/MS/MS System (Applied Biosystems). LC/MS/MS was performed by Dr Victoria Tyrrell (Professor O'Donnell Laboratory).

2.13 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6. Statistical significance was calculated using the appropriate statistical test (indicated in figure legend). A p value > 0.05 is indicated as not significant (ns). Significant results are indicated as follows: * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$).

Chapter Three

The role of Gata6 in the regulation of inflammation by resident peritoneal macrophages

3.1 Introduction

Macrophages are key sentinels and mediators of the inflammatory response against invading pathogens. They detect a wide array of PAMPs through their broad array of PRRs, of which the TLRs are the best characterised. The bacterial cell-wall component LPS is recognised by TLR4, which results in the expression of several hundred genes that amplify the initial inflammatory response, initiate the development of adaptive immunity, and exert antimicrobial activities. The production and release of soluble mediators by macrophages upon microbial recognition is a critical component of the host inflammatory response to pathogen infection. Cytokines are a diverse group of glycoproteins that are key mediators of the inflammatory response, which amplify the initial inflammatory response, initiate the development of adaptive immunity, promote antimicrobial effector functions, and promote the resolution of inflammation (Dinarello, 2007, Iwasaki and Medzhitov, 2010).

Members of the IL-1 family affect virtually all cells and organs and have a fundamental role in the initiation, amplification, and resolution of inflammation. Typically, IL-1 family members are pro-inflammatory, inducing the expression and synthesis of many effector proteins including cytokines/chemokines, COX-2, PLA2, and inducible nitric oxide synthase (iNOS) (Dinarello, 2009). Unsurprisingly, dysregulated IL-1 production has been implicated in the pathogenesis of many autoimmune, inflammatory diseases, and degenerative diseases (Dinarello et al., 2012, Carta et al., 2017, Hoffman et al., 2001). IL-1 β is a potent pro-inflammatory cytokine which mediates innate immunity and inflammation. As such, the production of IL-1 β is tightly-controlled, and generally requires two distinct signals. The first signal induces the expression of the biologically inactive precursor pro-IL-1 β , which is typically mediated through TLR signalling. Once synthesised, pro-IL-1 β needs to be proteolytically cleaved to the mature form, before it can be released and thereby exert its biological effects through binding to its cognate receptor, IL-1R. Typically, the processing of pro-IL-1 β is mediated by caspase-1, although numerous extracellular proteases have been reported to cleave pro-IL-1 β *in vivo* (Joosten et al., 2009, Kono et al., 2012). Caspase-1 itself is generally inactive in resting cells and requires activation by multimeric protein complexes called inflammasomes, which sense a plethora of endogenous and exogenous stimuli. The diverse array of stimuli which activate inflammasomes are generally referred to as the ‘second signal’ for IL-1 β processing

and release. Activation of the inflammasome and its subsequent assembly results in the auto-activation of caspase-1, which subsequently cleaves pro-IL-1 β into its biologically active form, which exits the cell and mediates signalling via IL-1R. In addition to the processing of IL-1 β (and IL-18), caspase-1 mediates a form of programmed cell death termed pyroptosis, characterised by cell swelling and plasma membrane rupture. Furthermore, recent studies have reported that in macrophages, pyroptotic cell death is required for the release of mature IL-1 β (Martín-Sánchez et al., 2016).

3.1.1 NLRP3 inflammasome activation

The NLRP3 inflammasome is the best characterised inflammasome to date, which has been demonstrated to be activated by a broad array of endogenous and exogenous stimuli (Latz et al., 2013). The diverse array of stimuli are structurally and molecularly diverse, which make it unlikely that they (all) bind directly to and activate the inflammasome. Therefore, the general consensus is that inflammasomes monitor cellular homeostasis (which is perturbed by activatory stimuli), and are activated when they detect a specific divergence from homeostasis, such as the recognition of DAMPs. Three models through which stimuli converge to activate the inflammasome have been proposed: lysosomal rupture, cytosolic K⁺ efflux, and ROS generation (Hornung and Latz, 2010, Petrilli et al., 2007, Tschoop and Schroder, 2010). However, reports on their relative contributions to NLRP3 activation have often been contradictory and a single unified model of inflammasome activation has yet to be agreed. More recently, it was reported that the release of potassium is the downstream convergence point for all NLRP3 activatory stimuli (Munoz-Planillo et al., 2013). However, it is currently unclear whether intracellular potassium levels modulate the activation of NLRP3 directly or whether another event correlates with low intracellular potassium concentrations is yet to be determined (Broz and Dixit, 2016).

Extracellular ATP is widely used *in vitro* as a potent activator of the NLRP3 inflammasome, which binds to P2X7 receptors resulting in the processing and release of IL-1 β . The release of ATP in resting macrophages is low, but substantially increased upon stimulation with TLR ligands (Cohen et al., 2013). Furthermore, both stressed and damaged cells release ATP into the extracellular space, which may lead to the

activation of the inflammasome. The release of IL-1 β from primary human monocytes stimulated with LPS alone was proposed to result from their endogenous release of ATP, leading to constitutive caspase-1 activity (Netea et al., 2009), but ATP-independent IL-1 β release from human monocytes following LPS stimulation has since been reported (Ward et al., 2010), and these discrepancies likely arise from different purification protocols for monocytes and reflect differential responses of different monocytic subsets. Macrophages are reported to release ATP into the extracellular milieu via pannexin-1 channels following TLR stimulation (Cohen et al., 2013). The extracellular ATP is catabolised by macrophages in a coordinated two-step process. First ATP is hydrolysed to AMP by the ectoenzyme CD39. AMP is subsequently dephosphorylated into adenosine by CD73. Thus, these ectoenzymes are thought to regulate anti-inflammatory signalling through the hydrolysis of extracellular ATP (which can activate the inflammasome), and through the sequential generation of adenosine, which is reported to terminate the synthesis of many pro-inflammatory cytokines (Antonioli et al., 2013). The expression of these two ectoenzymes can therefore determine the degradation of extracellular ATP and generation of adenosine in the extracellular milieu. Macrophages deficient of CD39 exhibit enhanced production of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF (Levesque et al., 2010). It has been suggested that CD39 has more profound anti-inflammatory effects than CD73, because the conversion of AMP to adenosine can occur in the absence of CD73 (Hamidzadeh and Mosser, 2016). However, recent studies suggest that the suppression of pro-inflammatory cytokine production through apoptotic cell clearance is dependent on CD73, and that CD73-deficient mice had enhanced neutrophil influx during a mild TLR4 peritonitis model (Murphy et al., 2017).

3.1.2 IL-1 β drives neutrophil recruitment *in vivo*

IL-1 β has a key function in the recruitment of neutrophils, and the IL-1-dependent peritoneal accumulation of neutrophils has frequently been used as an *in vivo* assay for IL-1 β production (Martinon et al., 2006, Guarda et al., 2009). Indeed, the influx of neutrophils into the peritoneum is markedly decreased in mice deficient of either IL-1 or IL-1R (Iyer et al., 2009). Furthermore, mice deficient of NLRP3 inflammasome components show similar perturbations to neutrophil recruitment, but noteworthy that the influx is less severely diminished compared to IL-1- or IL-1R-deficient mice

(Duewell et al., 2010). This differential response has largely been attributed to caspase-1-independent processing of IL-1 β and/or the contribution of IL-1 α (Dinarello, 2009).

3.1.3 Gata6 regulates the function and phenotype of pM ϕ

In the naïve mouse peritoneal cavity, mononuclear phagocytes can be subdivided by flow cytometry based upon the expression of F4/80 and CD11b, a major subset with an F4/80^{high}CD11b^{high}MHCII^{low} phenotype and a minor subset with an F4/80^{int-low}CD11b⁺MHCII^{high} phenotype (Dioszeghy et al., 2008, Rosas et al., 2007). The former was the classical resident pM ϕ , whereas the latter were tentatively called ‘DC-like’ cells, owing to superior antigen processing and presentation, and stimulation of T cell proliferation. However, during inflammation, this minor population could not be distinguished from recruited inflammatory monocyte-derived (Inf) M ϕ s due to a lack of definitive markers. The failure to identify these cells during inflammation led to ambiguity, and confusion of these cells with InfM ϕ s and DCs and as such, the composition of this population has been a source of controversy in recent years. The expression of CD11c in this population has led to some investigators to exclude this population (as CD11c is highly expressed in DCs), leading another group to describe two M ϕ subsets in the peritoneal cavity, a major population of ‘large peritoneal macrophages’ and a minor population, designated as ‘small peritoneal macrophages’ (Ghosn et al., 2010), corresponding to the classical resident pM ϕ s and DC-like cells described by our group. More recent studies have shown that classical DCs are found in the peritoneal F4/80^{low}MHCII⁺ subset (Bain et al., 2016). Transcriptional profiling of the F4/80^{int-low}CD11b⁺MHCII^{high} subset reveals that they have major differences to F4/80^{high} resident pM ϕ s, likely reflecting their distinct functions in the peritoneal cavity. In addition to CD11c, gene expression analysis of the F4/80^{int-low}CD11b⁺MHCII^{high} subset has shown that they selectively express the surface marker CD226, also known as DNAX accessory molecule-1 (Liao et al., 2016, Kim et al., 2016a). This marker enables the F4/80^{low}MHCII⁺ subset to be reliably distinguished from InfM ϕ s (CD11c⁻CD226⁻). Studies from our lab demonstrated that mice deficient of Flt3l (an important growth factor for DC) have a marked deficiency in the CD226⁺F4/80^{int-low}CD11b⁺MHCII^{high} subset (Liao et al., 2016).

Recent evidence suggests that distinct tissue-specific master regulators govern the phenotypic diversity and specialised functions of different tissue-specific

macrophages. Our research group as well as other groups identified the transcription factor *Gata6* as a critical regulator of a peritoneal macrophage-specific transcriptional program (Gautier et al., 2014, Okabe and Medzhitov, 2014, Rosas et al., 2014). Genetic deletion of *Gata6* in macrophages led to a dramatic reduction in the number of F4/80^{high} resident pMφs from approximately 50% in *Gata6*-WT mice to 20% in *Gata6*-KO^{mye} mice. The loss in resident pMφs in *Gata6*-KO^{mye} is accompanied by an increase in the number of eosinophils and F4/80^{int-low}MHCII^{high}CD226⁺ cells. However, studies in our lab indicate that the F4/80⁺Tim4⁺*Gata6*⁻ resident pMφs do not express CD226 or CD11c, suggesting that the remaining pMφs in *Gata6*-KO^{mye} mice are indeed resident macrophages corresponding to *Gata6*⁺F4/80^{high} in *Gata6*-WT mice.

3.2 Chapter hypothesis and aims

The hypothesis tested in this chapter is that Gata6 plays a fundamental role in the control of peritoneal macrophage inflammatory responses.

Aims

- To establish whether Gata6 regulates pro-inflammatory cytokine production
- To determine differential responses to various TLR ligands
- To evaluate the whether the release of IL-1 β is mature and dependent on the NLRP3 inflammasome
- To examine mechanisms of aberrant release of IL-1 β
- To determine whether *Gata6*-deficient pM ϕ s are predisposed to apoptosis both naïve and stimulated
- To evaluate whether purinogenic signalling and soluble factors contribute to IL-1 β release

3.3 Results

3.3.1 Dose-dependent IL-1 β release from LPS-stimulated *Gata6*-deficient pM ϕ s

Macrophages express a broad array of PRRs that detect pathogens and tissue-injury (sterile inflammation) via conserved PAMPs and DAMPs respectively. The TLRs represent the best characterised PRRs by which macrophages detect pathogens. To determine whether *Gata6*-deficient pM ϕ s have dysregulated inflammatory responses, pM ϕ s were stimulated with the archetypal TLR4 ligand, lipopolysaccharide (LPS), and the production of soluble mediators determined. The focus of this chapter is the regulation of IL-1 family member cytokines (Figure 3.1), and the production of other prototypical pro-inflammatory cytokines (TNF and IL-6) are discussed in Chapter Four.

In response to LPS, *Gata6*-deficient pM ϕ s secreted significantly more IL-1 β and IL-18 compared to pM ϕ s from *Gata6*-WT mice in a dose-dependent manner (Figure 3.1). The production of IL-1 β was detectable in *Gata6*-deficient pM ϕ s stimulated with 10 ng ml⁻¹ LPS and reached peak production at 100 ng ml⁻¹ LPS (Figure 3.1A). Conversely, IL-18 release from *Gata6*-deficient pM ϕ s was much lower than IL-1 β and was only significantly produced after stimulation with 100 ng ml⁻¹ LPS. Conversely, LPS-stimulated pM ϕ s from *Gata6*-WT mice did not produce significant amounts of IL-1 β or IL-18 compared to unstimulated controls.

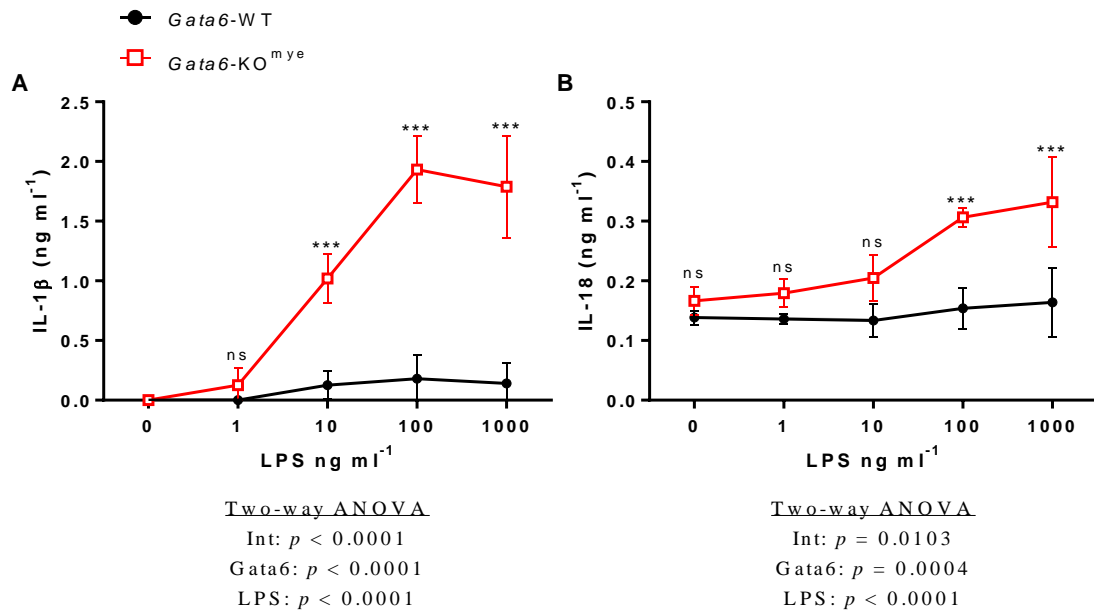


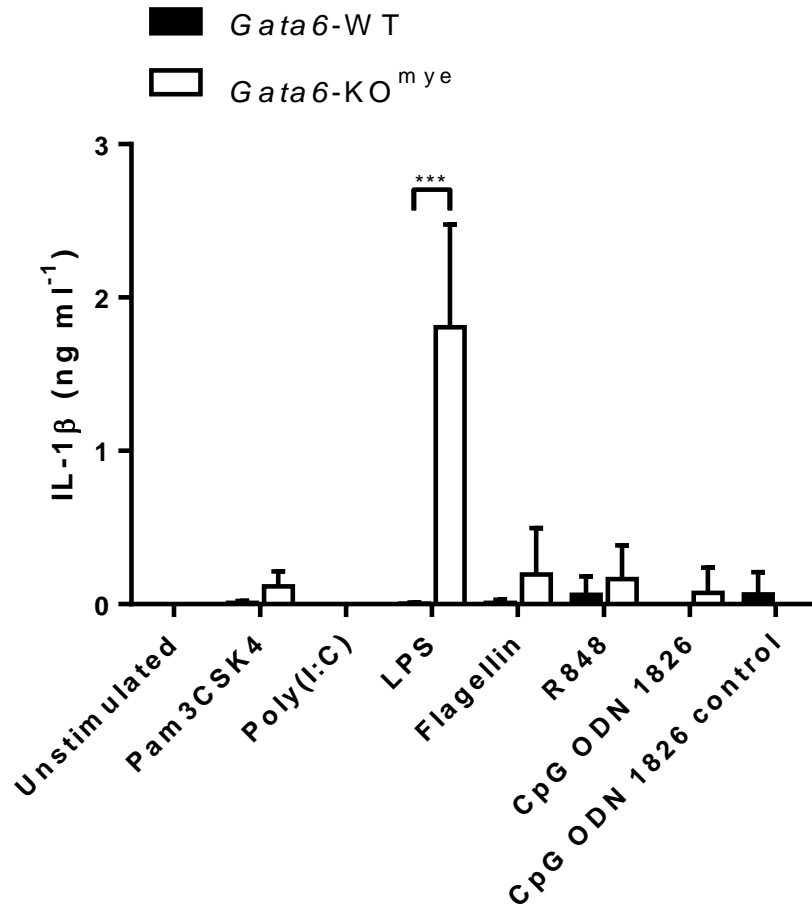
Figure 3.1 | *Gata6*-deficient pM ϕ s secrete IL-1 β in response to LPS alone.

Adherent peritoneal exudate cells (APECs) from *Gata6*-WT and *Gata6*-KO^{mye} were stimulated with LPS at 0, 1, 10, 100 or 1000 ng ml⁻¹ for 24 hours. Culture supernatants were collected and (A) IL-1 β and (B) IL-18 release measured by ELISA. Data are expressed as mean \pm S.D from three independent experiments. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Gata6, Gata6 effects; LPS, LPS concentration effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

3.3.2 Aberrant IL-1 β release from *Gata6*-deficient pM ϕ s is restricted to TLR4 stimulation

To further characterise the effect that *Gata6*-deficiency has on the production of IL-1 β release and the inflammatory response, an array of TLR ligands were used to stimulate pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} mice (Figure 3.2).

In contrast to LPS stimulation, which resulted in robust IL-1 β release from *Gata6*-deficient pM ϕ s ($p < 0.0001$), none of the other TLR ligands tested triggered a significant increase in IL-1 β production compared to unstimulated pM ϕ s (Figure 3.2).



Two-way ANOVA

Int: $p < 0.0001$

Gata6: $p < 0.0001$

Ligand: $p < 0.0001$

Figure 3.2 | IL-1 β release from *Gata6*-deficient pM ϕ s is restricted to LPS/TLR4 stimulation.

APECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were unstimulated or stimulated with Pam3CSK4 (500 ng ml⁻¹), Poly(I:C) (1 μ g ml⁻¹), LPS (100 ng ml⁻¹), Flagellin (100 ng ml⁻¹), R848 (1 μ g ml⁻¹), CpG ODN 1826 (5 μ M) or CpG ODN 1826 control (5 μ M) for 24 hours. Culture supernatants were collected 24 hours after stimulation and the release of IL-1 β measured by ELISA. Data are shown as mean \pm S.D from five independent experiments. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Gata6, Gata6 effects; Ligand, TLR ligand effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

3.3.3 *Gata6*-deficient pMφs exhibit enhanced and prolonged pro-inflammatory cytokine production in response to LPS

The production of soluble mediators by immune cells is tightly regulated, with defects in regulation underlying causes of a range of inflammatory disorders including arthritis, autoimmune diseases, and neurodegeneration (Sedger and McDermott, 2014, Clark, 2007, Tanaka and Kishimoto, 2012, Dinarello et al., 2012, Arnardottir et al., 2016). The resolution of inflammation is therefore a fundamental event of the immune response. To determine whether *Gata6* controls the inflammatory responses of pMφs the kinetics of pro-inflammatory cytokine and mediator production were investigated in response to LPS and zymosan, a TLR2/4 and dectin-1 ligand that has been reported to induce IL-1β release from macrophages (Figure 3.3).

In accordance with earlier observations, *Gata6*-WT pMφs stimulated with LPS resulted in only limited detection of IL-1β in the culture supernatants, whereas *Gata6*-deficient pMφs stimulated with LPS resulted in robust secretion of IL-1β (Figure 3.3). The stimulation of pMφs from *Gata6*-WT mice with zymosan (a ligand that can signal through MyD88-dependent and MyD88-independent pathways) resulted in robust secretion of IL-1β, with peak production detected at 24 hours, at which point IL-1β release was significantly higher than that from *Gata6*-deficient pMφs ($p = 0.0228$). (Figure 3.3). The kinetics of IL-1β release from LPS stimulated *Gata6*-KO^{mye} pMφs was comparable to both *Gata6*-WT and *Gata6*-KO^{mye} pMφs stimulated with zymosan (Figure 3.3).

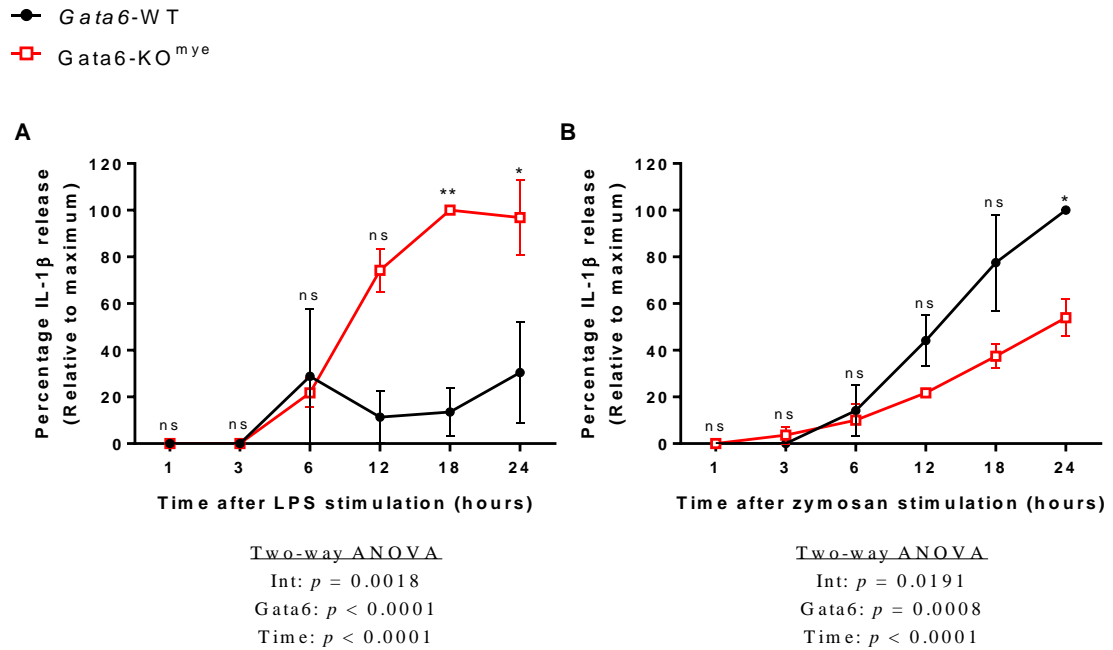


Figure 3.3 | *Gata6*-deficient pMφs have aberrant IL-1β release when stimulated with LPS.

APECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were stimulated with 100 ng ml⁻¹ LPS (A) or a 1:1 ratio zymosan (B) for the indicated time points. Supernatants were collected and analysed by ELISA for IL-1β release. Data are expressed as mean percentage of maximum release ± S.E.M of three independent experiments (Release of IL-1β from LPS-stimulated cells is expressed as a percentage of IL-1β released from *Gata6*-KO^{mye} APECs stimulated with LPS for 18 hours, and the release of IL-1β from zymosan-stimulated cells was expressed as a percentage of *Gata6*-WT APECs stimulated with zymosan for 24 hours). Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Gata6, Gata6 effects; Time, Ligand stimulation time effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

3.3.4 IL-1 β released by LPS-stimulated *Gata6*-deficient pM ϕ s is mature and caspase-1- and NLRP3-dependent

The production and release of IL-1 β is tightly regulated and typically requires two distinct signals (Martinon et al., 2002). The first signal induces the expression of pro-IL-1 β and NLRP3, whereas the second signal is required for the activation of the NLRP3 inflammasome. Activation of the inflammasome and subsequent assembly results in the activation of caspase-1, which then cleaves pro-IL-1 β into the mature biologically active form (Franchi et al., 2012, Mariathasan et al., 2006, Agostini et al., 2004).

Typically, the processing of pro-IL-1 β into the biologically active form requires cleavage by caspase-1. To test whether caspase-1 is critical for the processing and release of IL-1 β from *Gata6*-deficient pM ϕ s stimulated with LPS, pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} were stimulated with LPS in the presence or absence of the caspase-1 inhibitor, Ac-YVAD-cmk (YVAD), for 24 hours and the release of IL-1 β into culture supernatants measured by ELISA (Figure 3.4). The activation of caspase-1 is typically mediated by inflammasomes, of which the NLRP3 inflammasome is the best characterised to date. To determine whether the release of IL-1 β from *Gata6*-deficient pM ϕ s stimulated with LPS was dependent on the NLRP3 inflammasome, pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} were stimulated with LPS in the presence or absence of MCC950, a small molecule inhibitor of NLRP3 inflammasome activation (Figure 3.4). To address whether the IL-1 β release from LPS-stimulated *Gata6*-deficient pM ϕ s was the mature, processed form (p17), the cell culture supernatants from unstimulated or LPS-stimulated pM ϕ s were analysed by immunoblotting for IL-1 β (Figure 3.4).

The release of IL-1 β from LPS-stimulated *Gata6*-deficient pM ϕ s was significantly reduced when treated with 10 μ M and 40 μ M YVAD ($p < 0.0001$ and $p < 0.0001$ respectively). Similarly, the release of IL-1 β was significantly lower in *Gata6*-deficient pM ϕ s treated with LPS and MCC950 compared to stimulation with LPS alone ($p < 0.0001$). Immunoblotting showed that the IL-1 β detected in culture supernatants was mature, and that treatment with MCC950 attenuated the release of mature IL-1 β in LPS-stimulated pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} mice.

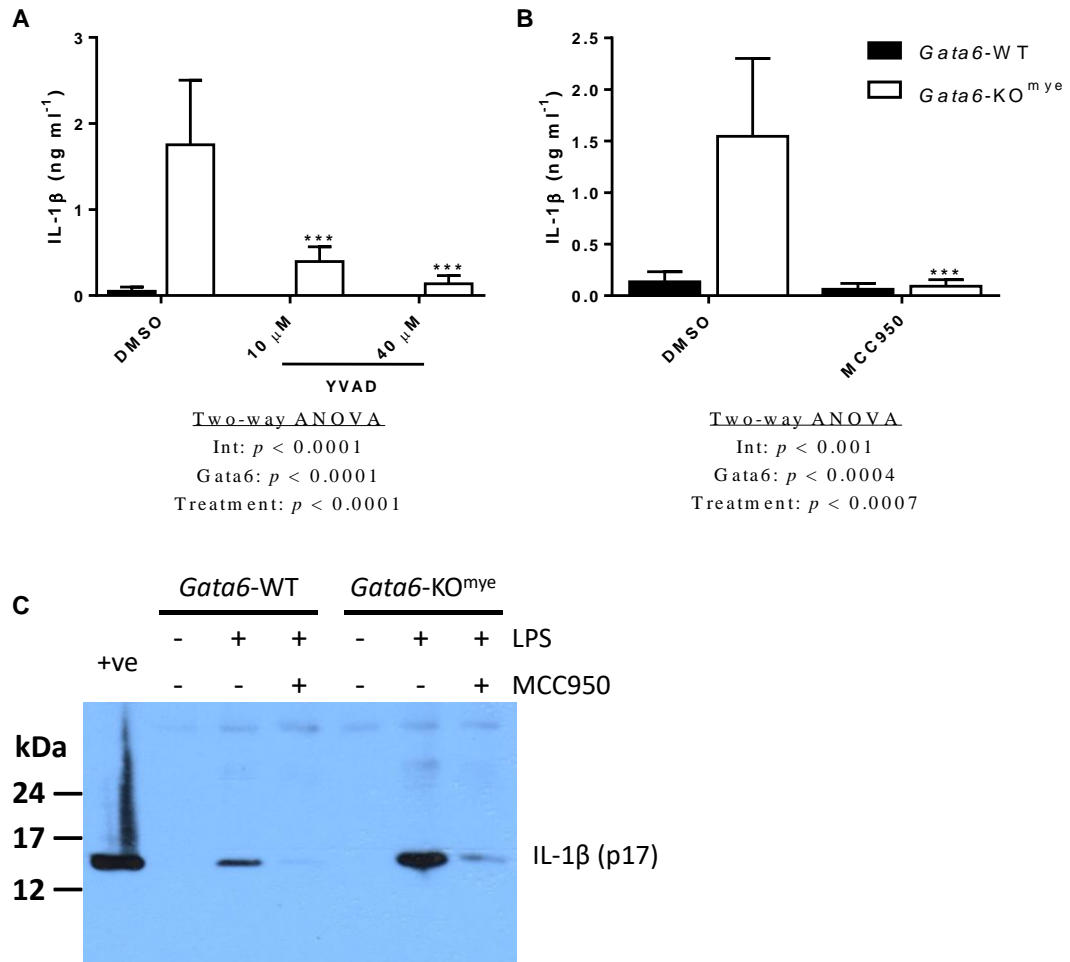


Figure 3.4 | The release of mature IL-1 β from LPS-stimulated *Gata6*-deficient pM ϕ is caspase-1- and NLRP3-dependent.

(A, B) APECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were stimulated with 100 ng ml⁻¹ LPS and either (A) Ac-YVAD-cmk or DMSO (vehicle control) or (B) DMSO or 1 μ M MCC950 for 24 hours. Culture supernatants were collected and IL-1 β release measured by ELISA. Data are expressed as mean \pm SD from five independent experiments. (C) Immunoblot analysis of IL-1 β from concentrated supernatants of APECs from *Gata6*-WT and *Gata6*-KO^{mye} stimulated with 100 ng ml⁻¹ LPS with or without 1 μ M MCC950 in serum-free medium for 18 hours. For a positive control (+ve) of mature IL-1 β , pM ϕ s were primed with 100 ng ml⁻¹ LPS for 3 hours followed by a 30 minute pulse with 5 mM ATP. (A, B) Data shows mean \pm S.D from five independent experiments. Statistical significance was determined by two-way ANOVA with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

3.3.5 Canonical inflammasome activation is comparable between *Gata6*-WT and *Gata6*-KO^{mye} pMφs

TLR4 mediated activation of NF-κB is required for the expression of both pro-IL-1β and NLRP3. Therefore, the role of *Gata6*-deficiency on the expression of pro-IL-1β and NLRP3 was determined in unstimulated and LPS stimulated pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice (Figure 3.5). To address whether *Gata6*-deficient pMφs have altered responses to conventional inflammasome activation, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice were primed with LPS for 3 hours, followed by treatment with either ATP or nigericin (Figure 3.5 C).

Western blot analysis of NLRP3 expression in unstimulated or LPS-stimulated pMφs showed that LPS treatment greatly enhanced the expression of NLRP3. Furthermore, there was no obvious difference in NLRP3 expression detected by immunoblotting in LPS-stimulated pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice. The production of IL-1β after LPS-primed pMφs were treated with ATP or Nigericin was comparable between *Gata6*-WT and *Gata6*-KO^{mye} pMφs ($p = 0.9914$ and $p = 0.7993$ respectively).

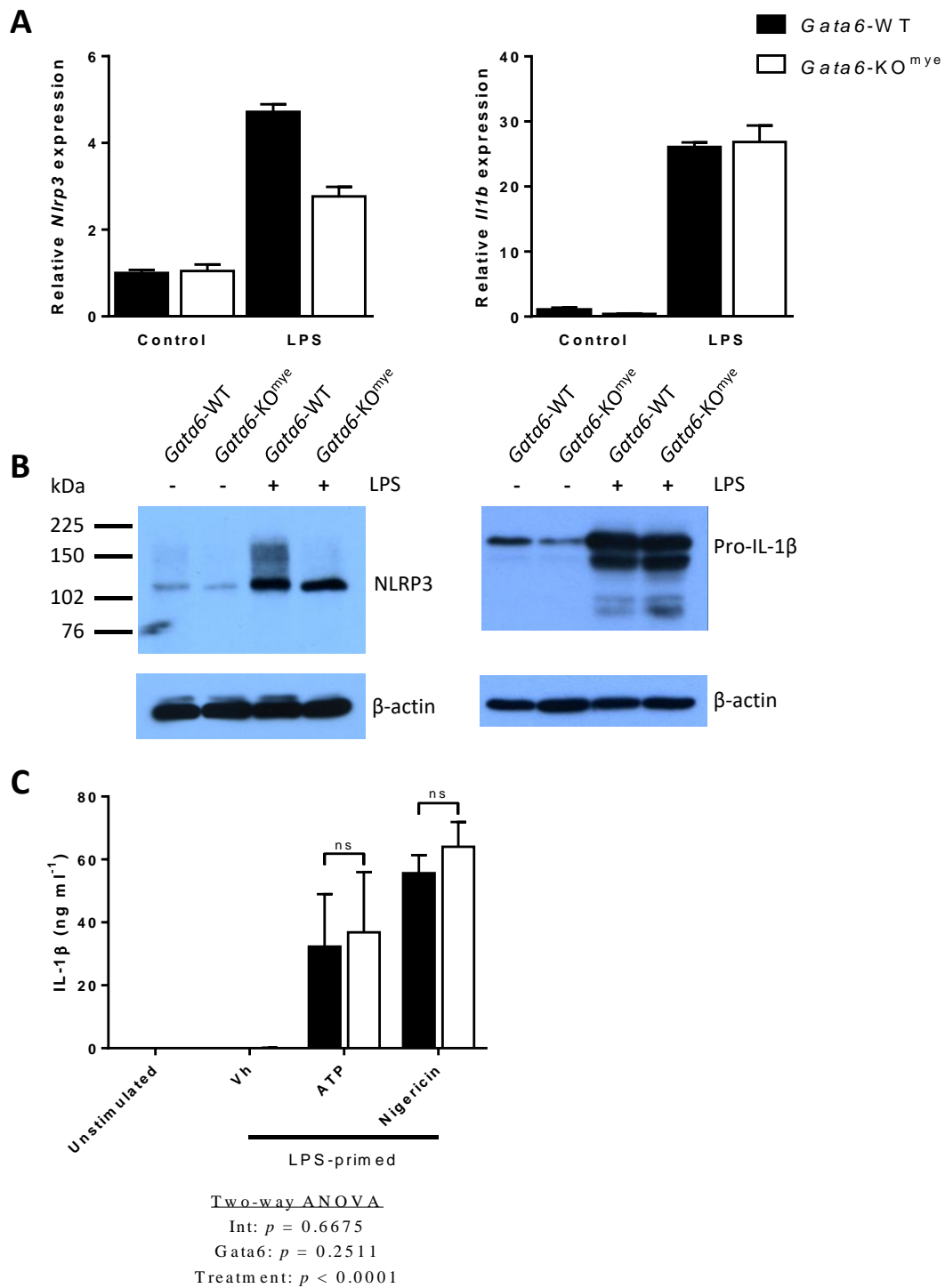


Figure 3.5 | *Gata6*-deficient pMφs have normal inflammasome function and NLRP3 expression.

(A) APECs from *Gata6*-WT and *Gata6*-K O^{mye} were unstimulated or stimulated with 100 ng ml⁻¹ LPS for 3 hours. The expression of *Il1b* and *Nlrp3* were determined by qPCR relative to the endogenous control gene *Ywhaz*. Error bars indicate the 95% confidence interval of three technical replicates. (B) Immunoblot analysis of NLRP3 and pro-IL-1β expression in pMφs from *Gata6*-WT or *Gata6*-K O^{mye} unstimulated or stimulated with 100 ng ml⁻¹ LPS for 6 hours. Data representative of two independent experiments. (C) APECs from *Gata6*-WT and *Gata6*-K O^{mye} were unstimulated or primed with 100 ng ml⁻¹ LPS for 3 hours, followed by a 30-minute pulse with vehicle

control (Vh), 5 mM ATP, or 20 μ M nigericin. Culture supernatants were collected and IL-1 β release measured by ELISA. Data shows mean \pm S.D from six independent experiments. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Gata6, Gata6 effects; Treatment, Treatment effects) with Tukeys multiple comparison post-test (*p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant).

3.3.6 *Gata6*-deficient pMφs exhibit no perturbations of NLRP3 inflammasome activatory pathways

Having established that mature IL-1 β release from LPS-stimulated *Gata6*-deficient pMφs was dependent on the NLRP3 inflammasome, I investigated possible mechanisms through which the loss of *Gata6* might promote aberrant inflammasome activation. Deficiencies in autophagy have been described to promote the processing and release of IL-1 β in response to LPS alone (Saitoh et al., 2008). Additionally, intracellular cyclic AMP (cAMP) and Ca²⁺ levels have been shown to modulate inflammasome activation (Lee et al., 2012). Furthermore, intracellular cAMP and Ca²⁺ levels are modulated by prostanoids, lipid mediators that are secreted by macrophages following TLR-signalling. Therefore, LPS-stimulated pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice were treated with chemical inhibitors targeting autophagy, cAMP and Ca²⁺ signalling pathways, and lipid signalling (Figure 3.6).

The release of IL-1 β from LPS-stimulated pMφs was not significantly affected by treatment with Dibutyryl-cAMP (DB), a cAMP analogue ($p = 0.2551$). Similarly, the release of IL-1 β from LPS-stimulated *Gata6*-deficient pMφs treatment with Rp-cAMPS (RP), an inhibitor of the activation of cAMP-dependent protein kinases by cAMP (cAMP signalling) was comparable to LPS treatment alone ($p = 0.9389$). Treatment of LPS-stimulated *Gata6*-deficient pMφs with the selective calcium chelator, BAPTA-AM, significantly reduced the secretion of IL-1 β compared to LPS treatment alone ($p = 0.0388$). The secretion of IL-1 β from LPS-stimulated *Gata6*-deficient pMφs treated with the prostacyclin analogue, beraprost, was significantly reduced by over 60% compared to LPS stimulation alone ($p = 0.0366$). Conversely, there was an almost two-fold increase in IL-1 β secretion from LPS-stimulated *Gata6*-deficient pMφs treated with the COX-1/2 inhibitor, indomethacin, compared to LPS stimulation alone ($p = 0.0010$). IL-1 β release from LPS-stimulated *Gata6*-deficient pMφs treated with Torin2, a compound that enhances autophagy, was significantly lower than LPS treatment alone ($p = 0.0110$). The secretion of IL-1 β from *Gata6*-deficient pMφs was comparable between LPS stimulation alone and treatment with either chloroquine or wortmannin ($p = 0.2838$ and $p = 0.1705$ respectively), which inhibit autophagy.

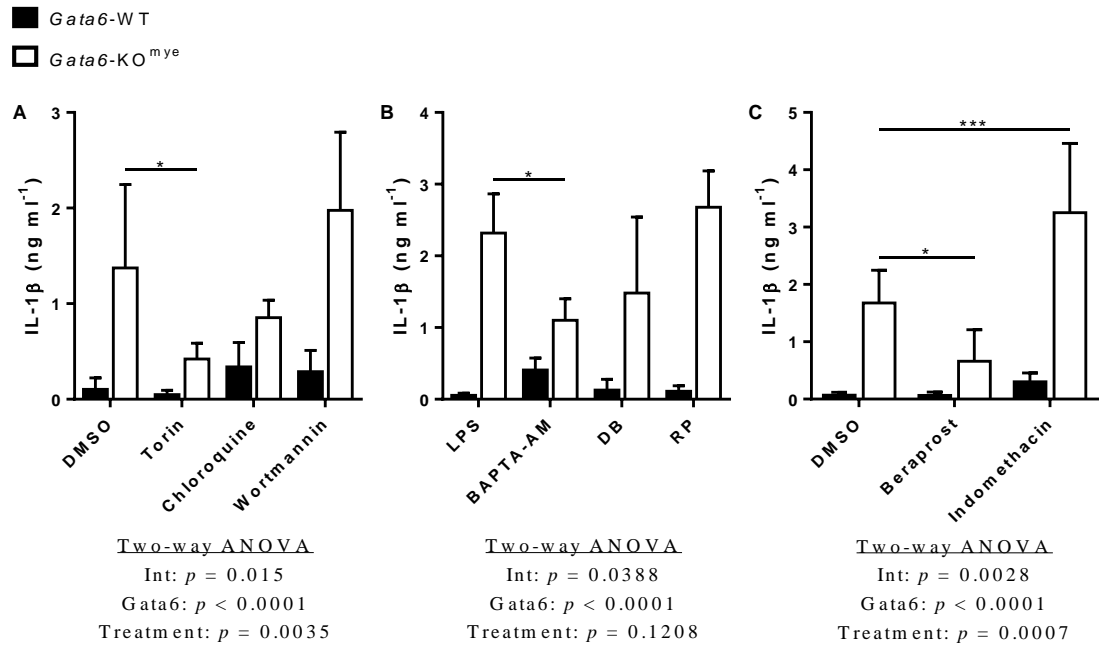


Figure 3.6 | *Gata6*-deficient pMφs exhibit no perturbations of NLRP3 inflammasome activatory pathways.

(A-C) APECs from *Gata6*-WT and *Gata6*-KO^{mye} were stimulated with LPS in the presence or absence of: (A) DMSO (vehicle control), 10 μM Torin2, 50 μM chloroquine, or 2.5 μM wortmannin; (B) LPS, 25 μM BAPTA-AM, 10 μM Dibutyryl-cAMP (DB), or 10 μM Rp-cAMPS (RP); (C) DMSO, 10 μM sodium beraprost, or 10 μM Indomethacin for 24 hours. Culture supernatants were collected and IL-1β release measured by ELISA. Data shows mean ± S.D from (A, C) five or (B) three independent experiments. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Gata6, Gata6 effects; Time, Ligand stimulation time effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

3.3.7 *Gata6*-deficient pMφs have dysfunctional mitochondria and enhanced reactive oxygen species generation

The NLRP3 inflammasome is activated by a wide array of endogenous and exogenous ligands. Dysfunctional mitochondria have been linked to the activation of the inflammasome, although this remains highly controversial. To examine whether *Gata6*-deficient pMφs have dysfunctional mitochondria, that might contribute to inflammasome activation and the release of mature IL-1β, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were analysed by flow cytometry for DHR123 and MitoSOX to determine the production of ROS and mitochondrial superoxide production respectively (Figure 3.7). *Gata6*-deficient pMφs had a significant increase in both DHR123 and MitoSOX staining compared to pMφs from *Gata6*-WT mice ($p = 0.0039$ and $p = 0.0078$ respectively). Furthermore, functional metabolic analyses on Tim4⁺-sorted pMφs showed that *Gata6*-deficient pMφs had enhanced basal oxidative phosphorylation but a lower spare respiratory capacity compared to pMφs from *Gata6*-WT mice (Figure 3.7 B). To test whether an increase in mitochondrial ROS was accounted for by the accumulation of dysfunctional mitochondria, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were stained with two mitochondrial-selective fluorescent dyes to distinguish total mitochondrial mass (MitoTracker Green) and respiring mitochondria (MitoTracker Red). *Gata6*-deficient pMφs that were either unstimulated or stimulated with LPS for 16 hours showed an increase in the frequency of dysfunctional mitochondria (MitoTracker Green^{high}, MitoTracker Red^{low}) compared to pMφs from *Gata6*-WT mice (Figure 3.7 D). Treatment of LPS-stimulated *Gata6*-deficient with MitoTempo, a ROS scavenger, pMφs significantly decreased the secretion of IL-1β release ($p < 0.0001$) (Figure 3.7 C).

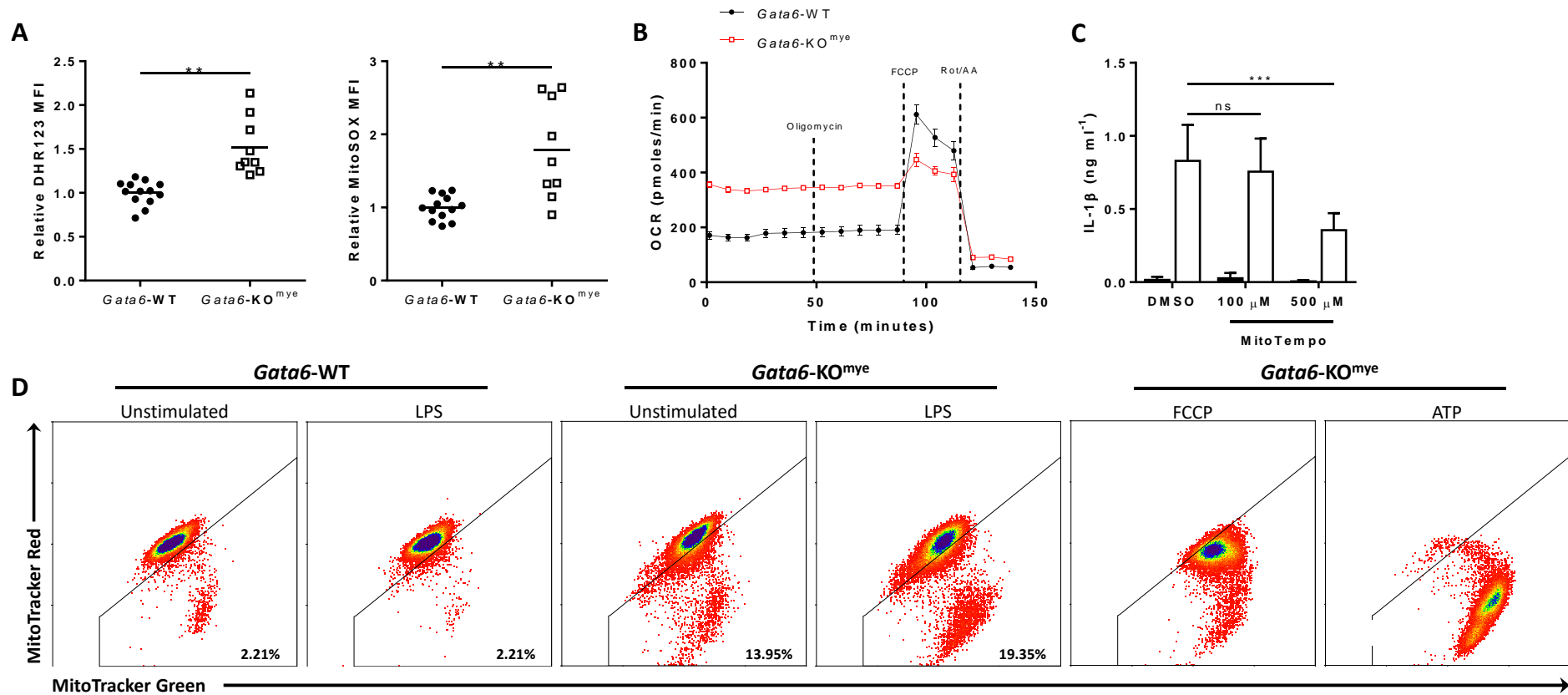


Figure 3.7 | *Gata6*-deficient pMφs have dysfunctional mitochondria and enhanced ROS.

(A) Scatter plots showing naïve DHR123 and MitoSOX MFI from naïve pMφs (CD11b⁺F4/80⁺MHC⁻) from *Gata6*-WT and *Gata6*-KO^{mye} mice. Each point shows DHR123 and MitoSOX MFI from individual mice (minus FMO control) expressed relative to *Gata6*-WT pMφs from two independent experiments. (B) Oxygen consumption rate (OCR) of pMφs from *Gata6*-WT and *Gata6*-KO^{mye} treated sequentially with oligomycin, FCCP, and Rotenone and antimycin A (Rot/AA) (mitochondrial stress test). OCRs were normalised to total protein in each well. Data representative

of two independent experiments. **(C)** APECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were stimulated with 100 ng ml⁻¹ LPS and either DMSO (vehicle control) or MitoTempo for 24 hours. Culture supernatants were collected and IL-1 β release measured by ELISA. Data are expressed as mean \pm S.D from six independent experiments. **(D)** Bivariate plot showing MitoTracker Green (binds to the lipid membrane of the mitochondria) and MitoTracker Red (accumulates in respiring mitochondria) in pM ϕ s (CD11b⁺F4/80⁺) from *Gata6*-WT and *Gata6*-KO^{mye} mice that were unstimulated or stimulated with 100 ng ml⁻¹ LPS for 16 hours. For a positive control of mitochondrial depolarization (loss of MitoTracker Red staining), pM ϕ s were treated with 100 μ M FCCP or 5 mM ATP. Statistical analysis was determined by **(A)** Wilcoxon matched-pairs signed rank test or **(C)** two-way ANOVA with Tukeys multiple comparison post-test (*p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant).

3.3.8 LPS induces cell death in *Gata6*-deficient pMφs

Recent studies have indicated that the release of mature IL-1 β from macrophages is coupled with the loss of plasma membrane integrity and cell death (Cullen et al., 2015, Liu et al., 2014). To address whether the release of IL-1 β in LPS-stimulated *Gata6*-deficient pMφs is accompanied by cell death, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were unstimulated or stimulated with LPS for 16 hours and the uptake of propidium iodide (PI) determined by flow cytometry (Figure 3.8).

The percentage of PI⁺ pMφs was comparable between unstimulated pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice ($p = 0.4193$). Conversely, stimulation with LPS significantly increased the percentage of PI⁺ pMφs from *Gata6*-KO^{mye} compared to unstimulated *Gata6*-deficient pMφs ($p = 0.0265$), and both unstimulated and LPS-stimulated pMφs from *Gata6*-WT mice ($p = 0.0033$ and $p = 0.0033$ respectively).

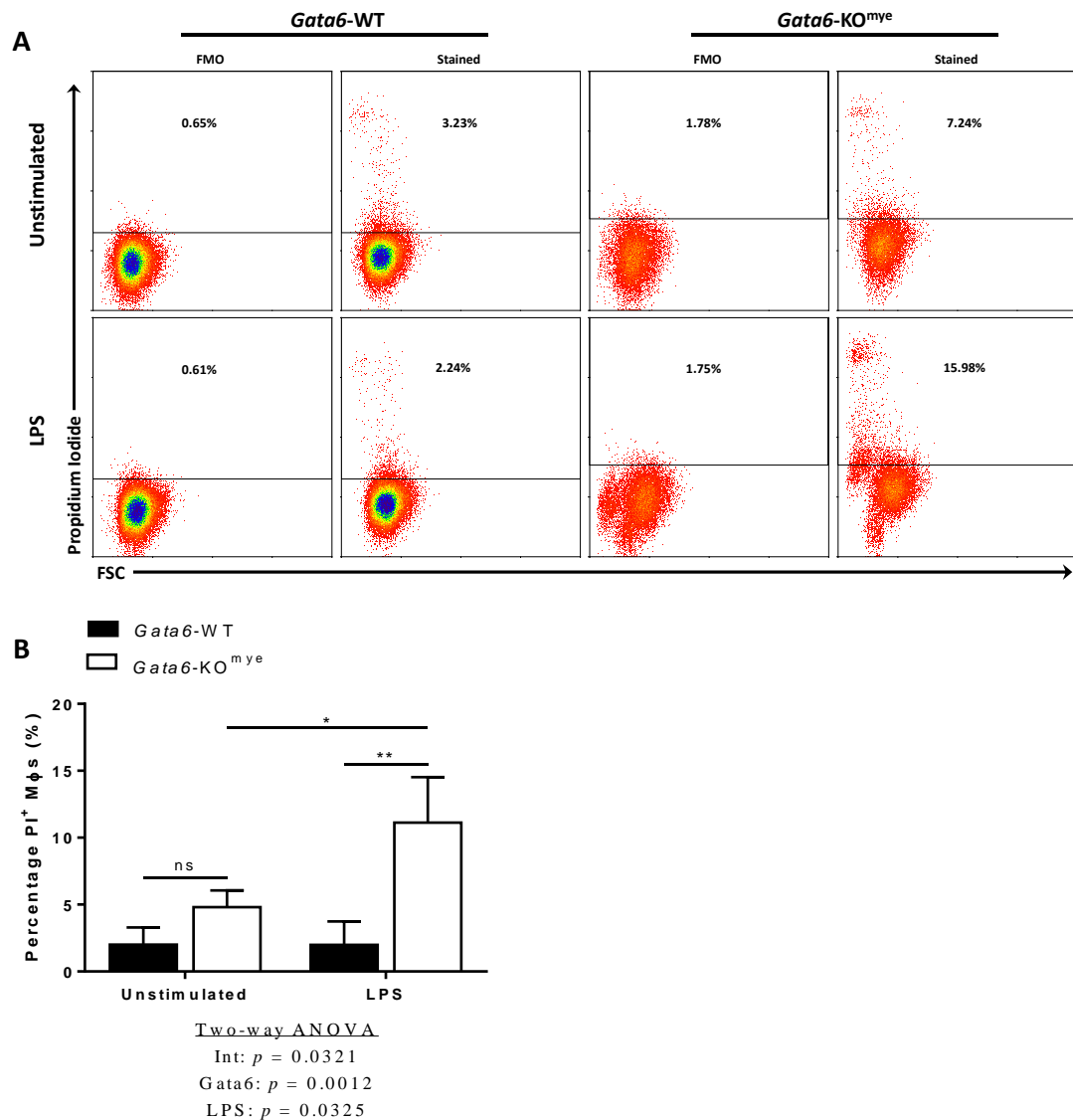


Figure 3.8 | LPS-stimulation induces cell death in *Gata6*-deficient pMφs.

(A) Representative flow cytometry bivariate plot showing the uptake of propidium iodide (PI) in pMφs (gated by CD11b⁺F4/80⁺) from *Gata6*-WT or *Gata6*-KO^{mye} mice that were either unstimulated or stimulated with 100 ng ml⁻¹ LPS *ex vivo* for 16 hours. Numbers indicate percentage PI⁺ pMφs. (B) Percentage PI positive pMφs were determined using FMO controls. Data are expressed as mean ± S.D from three independent experiments. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Gata6, Gata6 effects; LPS, LPS effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

3.3.9 Inhibition of Caspase-1 and NLRP3 fails to prevent cell death

Pyroptosis is typically mediated by caspase-1, which cleaves gasdermin d leading to the formation of plasma membrane pores and subsequent plasma membrane rupture. LPS induces a significant increase in cell death of *Gata6*-deficient pMφs, I next sought to determine whether inhibition of caspase-1 and the NLRP3 inflammasome (which inhibit the release of IL-1β) would prevent cell death, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were unstimulated or stimulated with LPS for 16 hours in the presence or absence of Ac-YVAD or MCC950 (Figure 3.9).

Interestingly, inhibition of caspase-1 or inhibition of NLRP3 inflammasome activation did not attenuate cell death in LPS-stimulated *Gata6*-deficient pMφs (Figure 3.9). Preliminary experiments suggest that the inhibitors themselves did not induce cell death as unstimulated *Gata6*-deficient pMφs in the presence or absence of inhibitors had comparable cell death (Supplementary Figure 1).

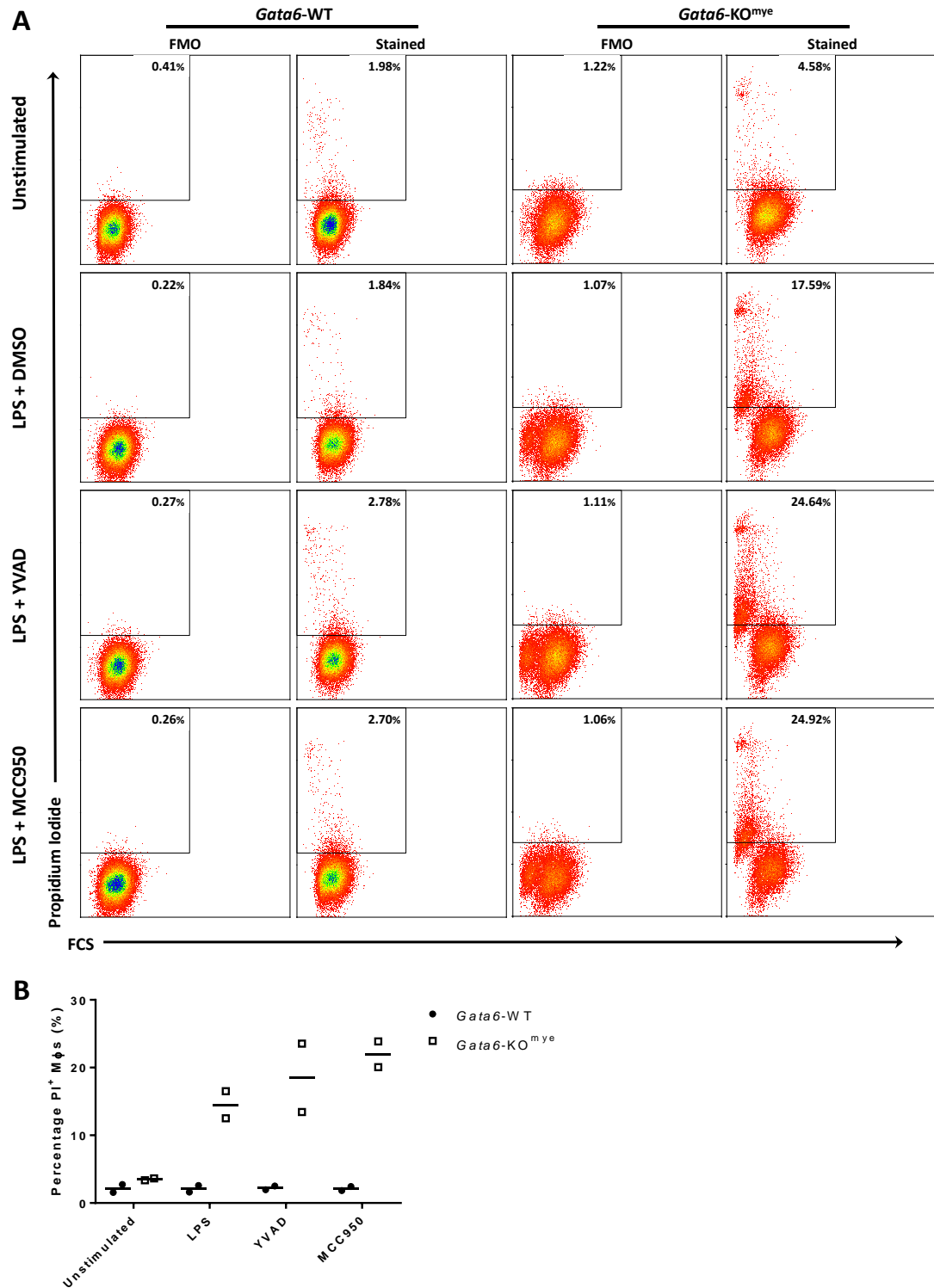


Figure 3.9 | Inhibition of caspase-1 and NLRP3 inflammasome activation does not prevent cell death.

(A) Bivariate plot showing the uptake of propidium iodide (PI) in pMφs (gated by CD11b⁺F4/80⁺) from *Gata6*-WT or *Gata6*-KO^{mye} mice that were either unstimulated or stimulated with 100 ng ml⁻¹ LPS with the presence of DMSO (vehicle control), 40 μM YVAD or 1 μM MCC950 *ex vivo* for 16 hours. Numbers indicate percentage PI⁺ pMφs. Data is representative of two independent experiments. (B) Scatter plot showing percentage PI⁺ pMφs from two independent experiments.

3.3.10 Naïve *Gata6*-deficient pMφs are not apoptotic

The dramatic reduction of peritoneal macrophages in *Gata6*-KO^{mye} mice has been attributed to increased apoptosis under homeostatic conditions (Gautier et al., 2014), whereas another study attributed the reduction to increased accumulation of pMφs in the omentum (Okabe and Medzhitov, 2014).

To investigate whether naïve *Gata6*-deficient pMφs have elevated apoptosis during the steady state, freshly isolated pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were analysed by flow cytometry for cleaved caspase-3 (active), which is indicative of cells undergoing terminal apoptosis (Figure 3.10). Furthermore, naïve pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were stained with Annexin V (Figure 3.10).

In contrast with previous findings (Gautier et al., 2014), the percentage of caspase-3 positive *Gata6*-deficient pMφs was comparable to those of *Gata6*-WT mice ($p = 0.8514$) (Figure 3.10 C). Similarly, Annexin V staining showed no discernible difference between naïve pMφ from *Gata6*-WT and *Gata6*-KO^{mye} mice (Figure 3.10 D-E).

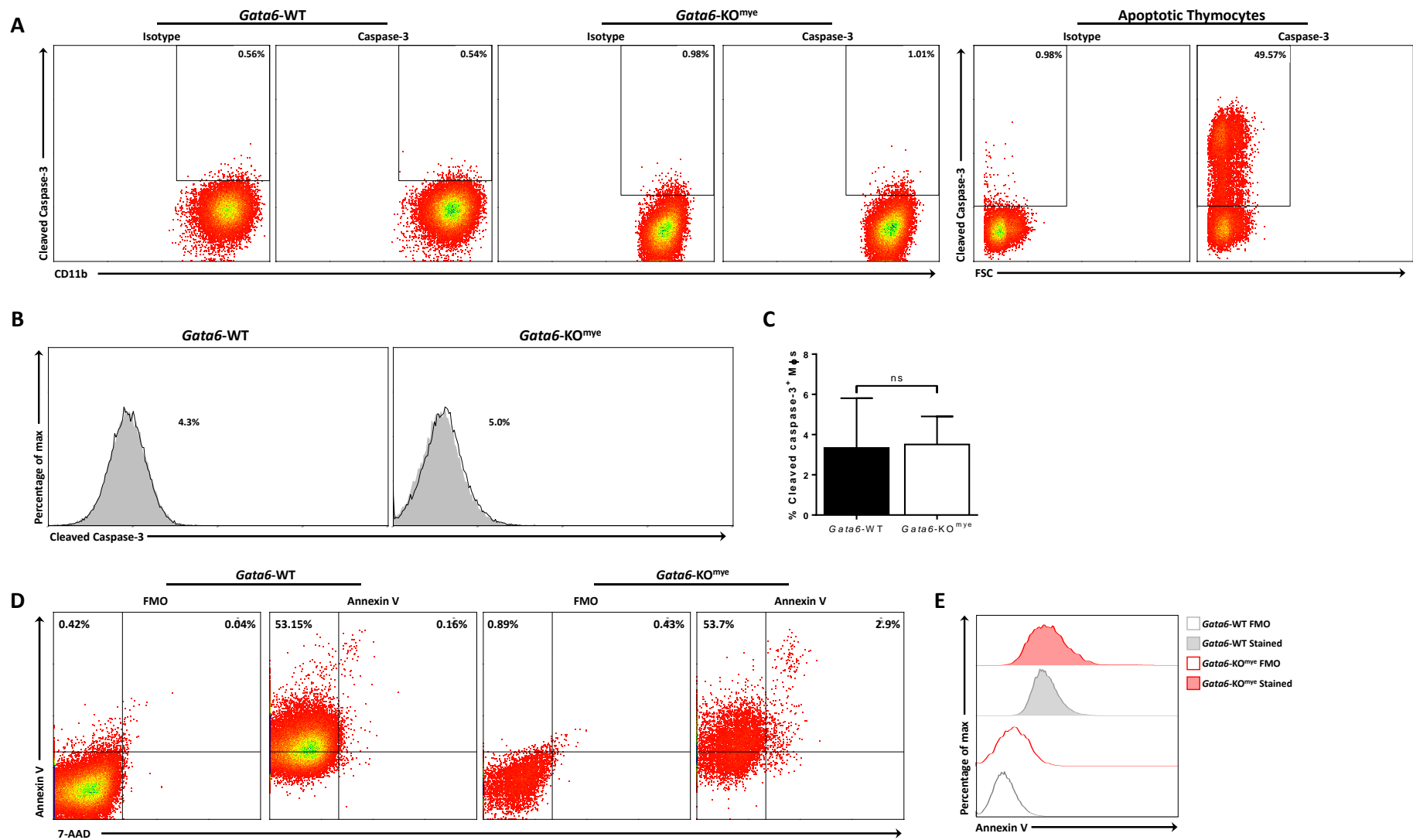


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Figure 3.10 | Naïve pMφs from *Gata6*-KO^{mye} mice are not significantly predisposed to apoptosis.

(A) Representative bivariate plots showing cleaved caspase-3 in naïve pMφs (CD11b⁺F4/80⁺) from *Gata6*-WT or *Gata6*-KO^{mye} mice. Thymocytes treated with dexamethasone for 4 hours are included as a positive control for apoptosis. (B) Representative overlay histograms showing cleaved caspase-3 in pMφs (CD11b⁺F4/80⁺) (shaded histogram: isotype control). Number indicates percentage positive cells. (C) Bar chart shows mean percentage caspase-3 positive pMφs ± S.D from 10 *Gata6*-WT and 11 *Gata6*-KO^{mye} mice from two independent experiments. Caspase-3 positive pMφs was determined using subtraction histograms of isotype control and stained cells. (D) Bivariate plot showing Annexin V staining in naïve pMφs (CD11b⁺F4/80⁺) from *Gata6*-WT or *Gata6*-KO^{mye} mice from one experiment. (E) Histogram showing cleaved Annexin V in naïve *Gata6*-WT or *Gata6*-KO^{mye} pMφs (CD11b⁺F4/80⁺) (Grey, WT; Red, KO; shaded, stained; empty, FMO). Statistical significance was determined by unpaired t test with Welch's correction (*p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant).

3.3.11 LPS induces apoptosis in *Gata6*-deficient pMφs

Having determined that cell death in LPS-stimulated *Gata6*-deficient pMφs was independent of caspase-1 and NLRP3 activation and that freshly isolated *Gata6*-deficient pMφs were not apoptotic, I next sought to determine whether LPS-stimulation would induce apoptosis in *Gata6*-deficient pMφs. To address this, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were either unstimulated or stimulated with LPS and analysed by flow cytometry for cleaved caspase-3 (Figure 3.11).

The percentage of caspase-3⁺ pMφs was comparable between unstimulated pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice ($p = 0.9983$). Conversely, stimulation with LPS significantly increased the percentage of caspase-3⁺ pMφs from *Gata6*-KO^{mye} compared to unstimulated *Gata6*-deficient pMφs ($p = 0.0003$), and both unstimulated and LPS-stimulated pMφs from *Gata6*-WT mice ($p = 0.0003$ and $p = 0.004$ respectively).

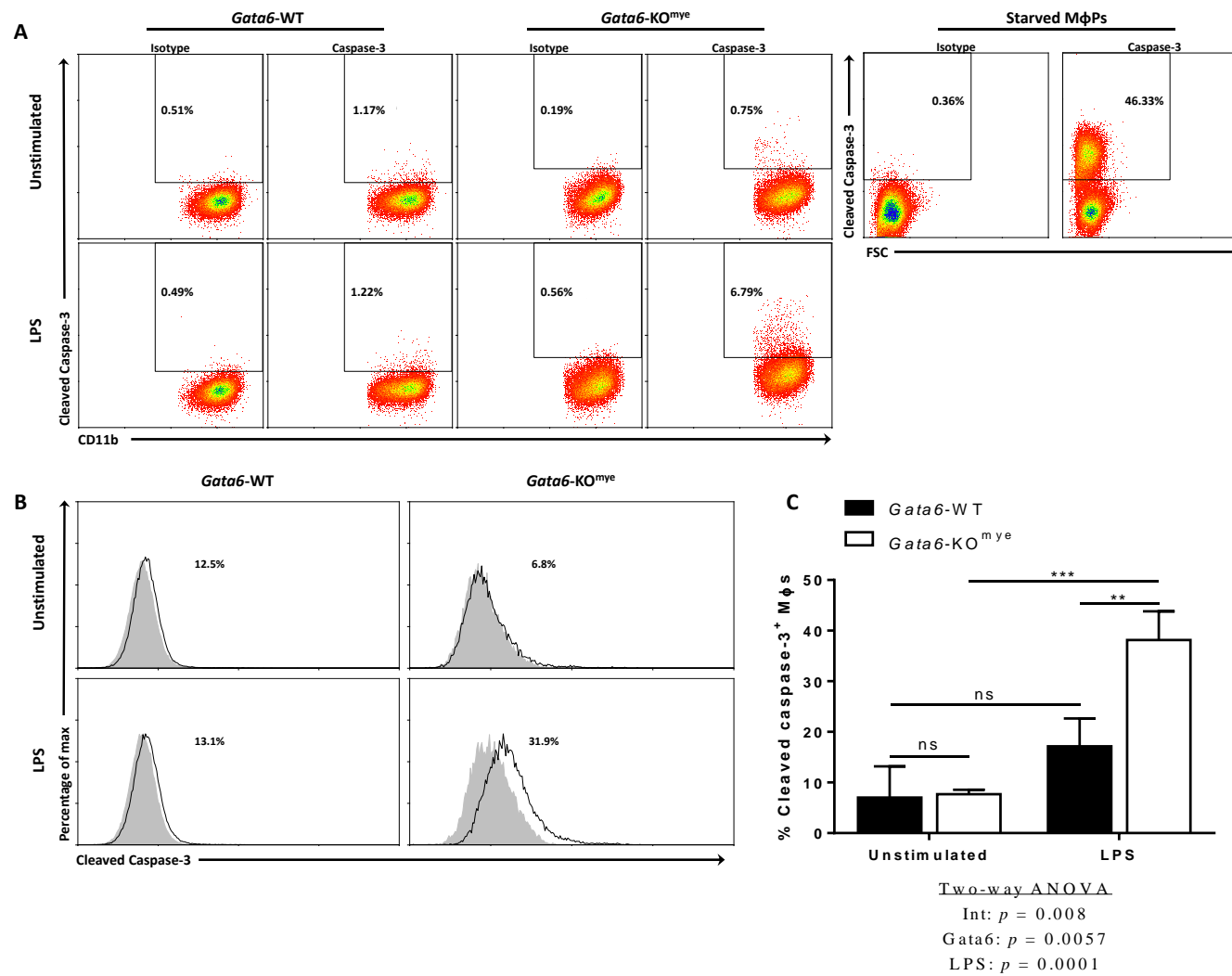


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Figure 3.11 | Caspase-3 activation is increased in LPS-stimulated *Gata6*-deficient pMφs.

(A) Representative bivariate plots showing cleaved caspase-3 in pMφs (CD11b⁺F4/80⁺) from *Gata6*-WT or *Gata6*-KO^{mye} mice that were unstimulated or stimulated with 100 ng ml⁻¹ LPS *ex vivo* for 16 hours. Starved macrophage precursors (MφPs) were included as a positive control for apoptosis. (B) Representative overlay histograms showing cleaved caspase-3 in pMφs (CD11b⁺F4/80⁺) (shaded histogram: isotype control) (C) Percentage cleaved caspase-3 positive pMφs was determined using subtraction histograms of isotype control and stained cells. Data are expressed as mean ± S.D from three independent experiments. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; *Gata6*, *Gata6* effects; LPS, LPS effects) with Tukeys multiple comparison post-test (*p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant).

3.3.12 Differential TLR signalling required for aberrant IL-1 β release

The release of IL-1 β from *Gata6*-deficient pM ϕ s was restricted to stimulation with the TLR4 ligand LPS (Figure 3.2). The production and release of mature IL-1 β requires both the induction of pro-IL-1 β expression and the activation of inflammasomes, which culminate in the cleavage and release of IL-1 β . Therefore, the incapacity of *Gata6*-deficient pM ϕ s stimulated with other TLR ligands to secrete IL-1 β may be a consequence of their failure to induce pro-IL-1 β expression, or that downstream signalling cascades fail to activate the inflammasome (either directly or indirectly). Understanding how LPS differentially induces IL-1 β release from *Gata6*-deficient pM ϕ s may provide insight into the molecular mechanisms governing aberrant release. The selective expression of different TLR receptors in pM ϕ s may account for the failure to induce IL-1 β secretion in *Gata6*-deficient pM ϕ s (Supplemental Figure 3), which is supported by the failure of some ligands to induce the production of other pro-inflammatory cytokines. Microarray expression analysis indicated that both TLR2 and TLR4 were highly expressed in pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye}. Furthermore, pM ϕ s stimulated with the TLR1/2 ligand Pam3CSK4 resulted in robust secretion of IL-6 (Chapter 4). Consequently, Pam3CSK4 was selected for further investigation into the differential mechanisms of LPS-induced secretion of IL-1 β from *Gata6*-deficient pM ϕ s. To ascertain whether Pam3CSK4 can induce the expression of pro-IL-1 β , pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} were either unstimulated or stimulated with either LPS or Pam3CSK4 for 6 hours and the expression of pro-IL-1 β determined by flow cytometry (Figure 3.12).

The percentage of pM ϕ s from *Gata6*-WT mice expressing pro-IL-1 β was comparable when cells were stimulated with either LPS or Pam3CSK4 ($p > 0.9999$). Similarly, there was no significant difference in the percentage of *Gata6*-deficient pM ϕ s expressing pro-IL-1 β stimulated with either LPS or Pam3CSK4 ($p = 0.4356$). The percentage of LPS-stimulated pM ϕ s expressing pro-IL-1 β was significantly lower in pM ϕ s from *Gata6*-KO^{mye} compared to *Gata6*-WT mice ($p = 0.0014$). Likewise, there was a significant reduction in the percentage of unstimulated pM ϕ s from *Gata6*-KO^{mye} expressing pro-IL-1 β compared to those from *Gata6*-WT mice ($p = 0.005$). Conversely, there was no significant difference in the percentage of Pam3CSK4-

stimulated pMφs expressing pro-IL-1 β from either *Gata6*-WT or *Gata6*-KO^{mye} ($p = 0.0821$).

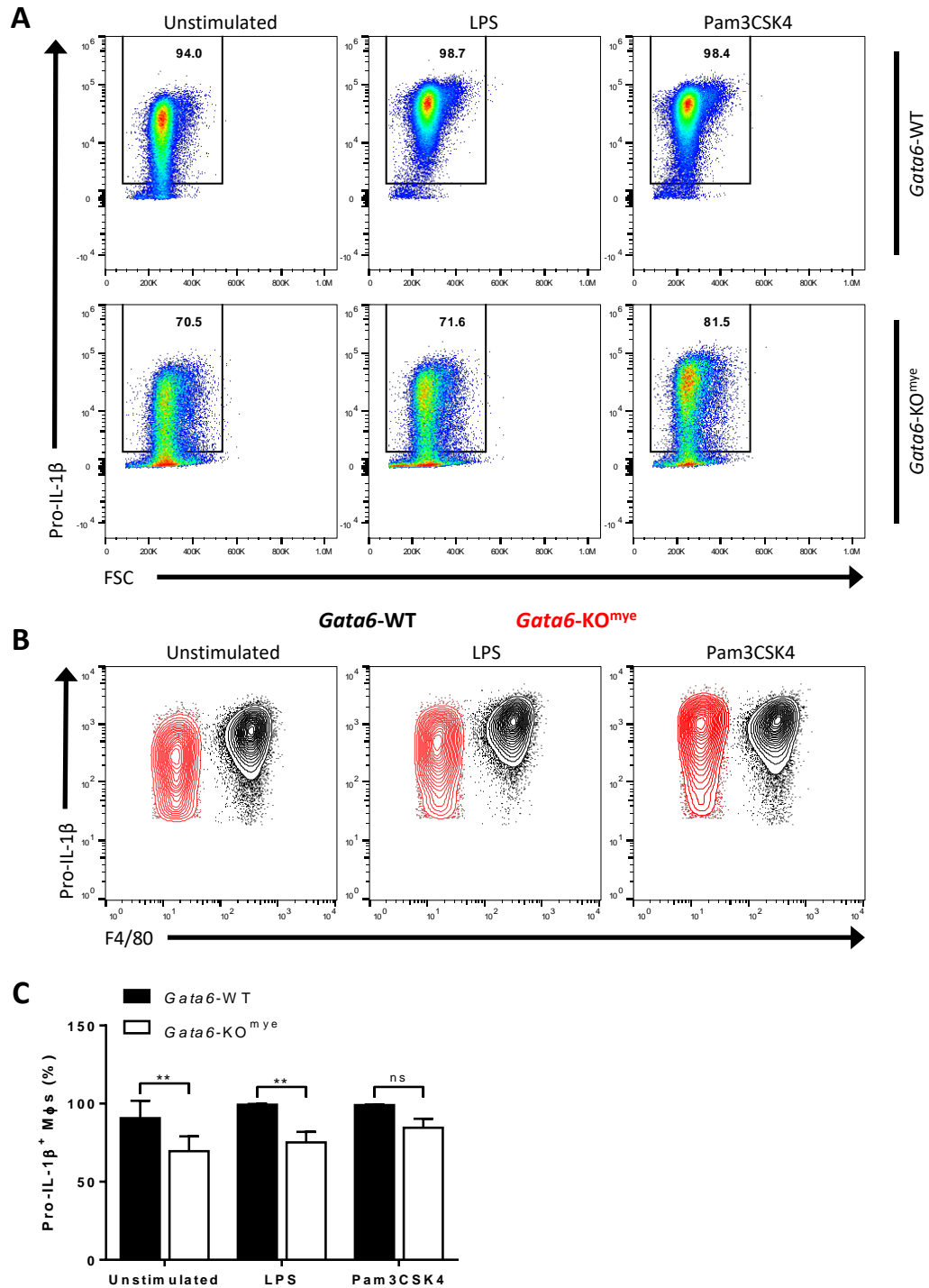


Figure 3.12 | Pam3CSK4 induces robust pro-IL-1 β production.

Total PECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were unstimulated or stimulated with either 100 ng ml⁻¹ LPS or 500 ng ml⁻¹ Pam3CSK4 for 6 hours. **(A)** Representative bivariate plot showing pro-IL-1 β expression in F4/80⁺Tim4⁺ pM ϕ s. The percentage pro-IL-1 β positive cells is indicated on each plot. **(B)** Representative overlay 5% contour plots with outliers of IL-1 β -expressing F4/80⁺Tim4⁺ pM ϕ s from *Gata6*-WT (black contours, F4/80^{high}) and *Gata6*-KO^{mye} (red contours, F4/80^{low}). **(C)** Bar chart shows percentage pro-IL-1 β positive pM ϕ s. Data shows mean \pm S.D from four independent experiments. Statistical significance was determined by two-way

ANOVA with Tukey's multiple comparisons post-test (* $p < 0.05$, ** <0.01 , ns = not significant).

3.3.13 Cell death is restricted to LPS stimulation in *Gata6*-deficient pMφs

The capacity of Pam3CSK4 to induce the expression of pro-IL-1 β but not secretion of mature IL-1 β from *Gata6*-deficient pMφs, indicated that downstream pathways differentially induced by Pam3CSK4 and LPS were responsible for the specific release of IL-1 β from *Gata6*-deficient pMφs stimulated with LPS but not Pam3CSK4. In response to LPS, the percentage of necrotic (PI⁺) *Gata6*-deficient pMφs was significantly higher compared to both unstimulated pMφs and unstimulated or LPS-stimulated pMφs from *Gata6*-WT mice (Figure 3.8). Consequently, I hypothesized that as Pam3CSK4 does not induce the secretion of IL-1 β in *Gata6*-deficient pMφs, cell death will be comparable to unstimulated cells. To address this, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were either unstimulated or stimulated with either LPS or Pam3CSK4 for 16 hours and the percentage of dead cells determined by flow cytometry (Figure 3.13).

There was a marked increase in cell death in *Gata6*-deficient pMφs stimulated with LPS compared to unstimulated pMφs, in accordance with previous observations ($p = 0.0009$). Conversely, pMφs stimulated with Pam3CSK4 had comparable cell death to unstimulated controls ($p = 0.9537$) (Figure 3.13). Similarly, the percentage of PI⁺ pMφs from *Gata6*-WT mice was comparable between unstimulated and either LPS or Pam3CSK4 stimulation ($p > 0.9999$ and $p = 0.9998$ respectively).

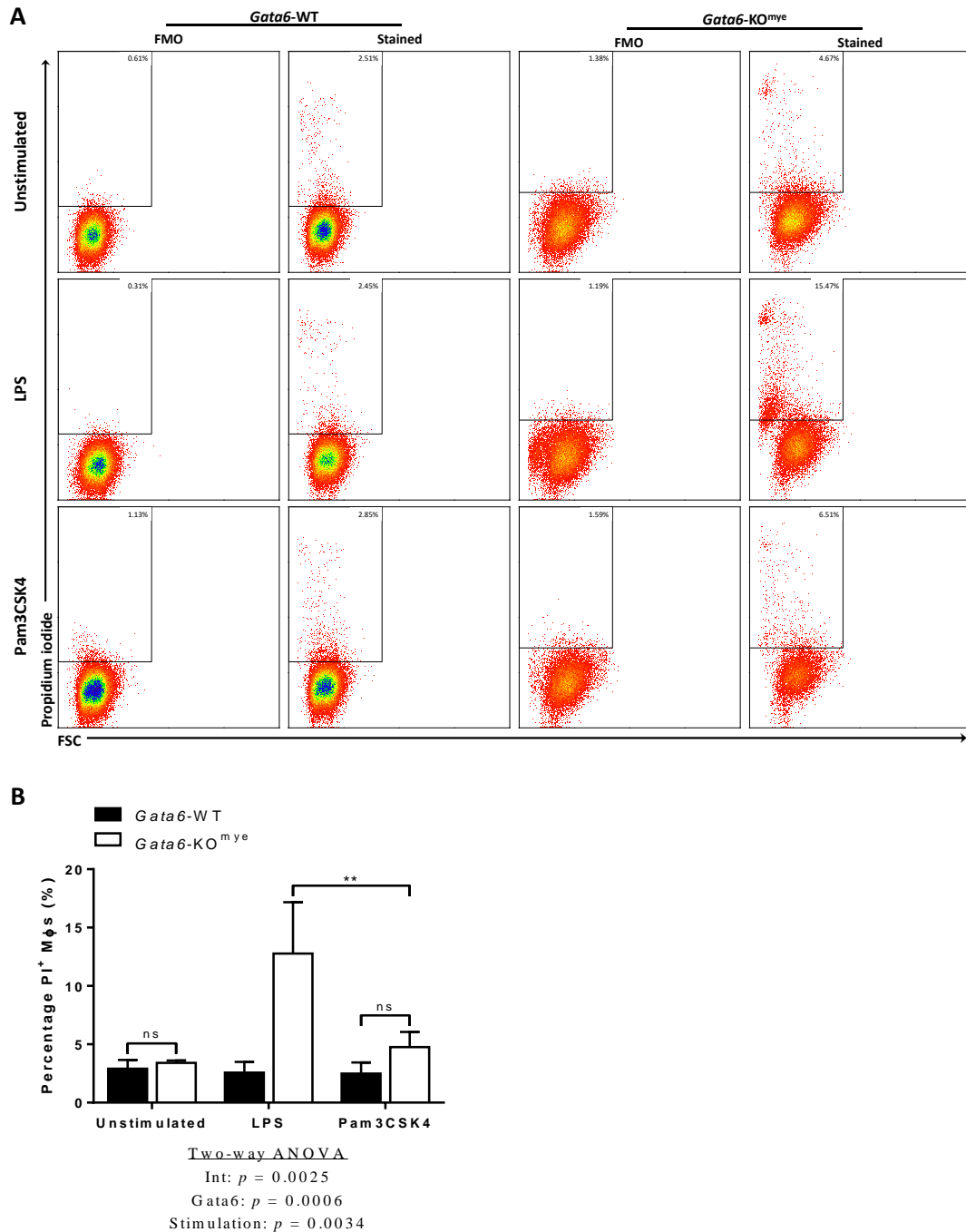


Figure 3.13 | Cell death of *Gata6*-deficient pMφs is restricted to stimulation with LPS.

(A) Bivariate plot showing the uptake of propidium iodide (PI) in pMφs (gated by Tim4⁺F4/80⁺) from *Gata6*-WT or *Gata6*-KO^{mye} mice that were either unstimulated or stimulated with 100 ng ml⁻¹ LPS or 500 ng ml⁻¹ Pam3CSK4 for 16 hours. The percentage PI⁺ cells is indicated on each plot. (B) Bar chart shows mean PI⁺ pMφs ± S.D from three independent experiments. Percentage PI⁺ pMφs was determined using FMO controls. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; *Gata6*, *Gata6* effects; Stimulation, Stimulation effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

3.3.14 Cell death and IL-1 β release from *Gata6*-deficient pM ϕ s are significantly reduced when co-cultured with pM ϕ s from *Gata6*-WT mice

To investigate whether the release of IL-1 β from LPS-stimulated *Gata6*-deficient pM ϕ s was a cell autonomous defect, or whether the release of soluble factors or contact-dependent mechanisms could modulate the release of IL-1 β , co-cultured pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} were stimulated with LPS and the release of IL-1 β determined by ELISA (Figure 3.14). Additionally, cell death was determined by PI staining in monocultures and co-cultures unstimulated and LPS-stimulated pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} mice.

The secretion of IL-1 β from LPS-stimulated *Gata6*-KO^{mye} monocultures seeded 1×10^5 cells was significantly higher than those containing 0.5×10^5 *Gata6*-deficient pM ϕ s or co-cultured pM ϕ s ($p = 0.0275$ and $p < 0.0001$ respectively). Co-cultured pM ϕ s released significantly less IL-1 β than monocultures containing 0.5×10^5 *Gata6*-deficient pM ϕ s ($p = 0.0181$). Conversely, the release of IL-1 β from co-cultured pM ϕ s was not significantly different to monocultures containing 0.5×10^5 pM ϕ s from *Gata6*-WT mice ($p = 0.4843$) (Figure 3.14).

Similar to previous observations, the percentage of PI⁺ *Gata6*-deficient pM ϕ s was significantly higher than unstimulated *Gata6*-deficient pM ϕ s and both unstimulated and LPS-stimulated pM ϕ s from *Gata6*-WT mice. Strikingly, the percentage of PI⁺ *Gata6*-deficient pM ϕ s stimulated with LPS was significantly reduced when co-cultured with pM ϕ s from *Gata6*-WT mice.

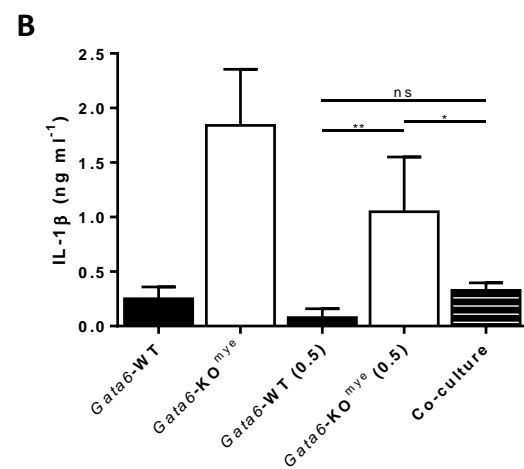
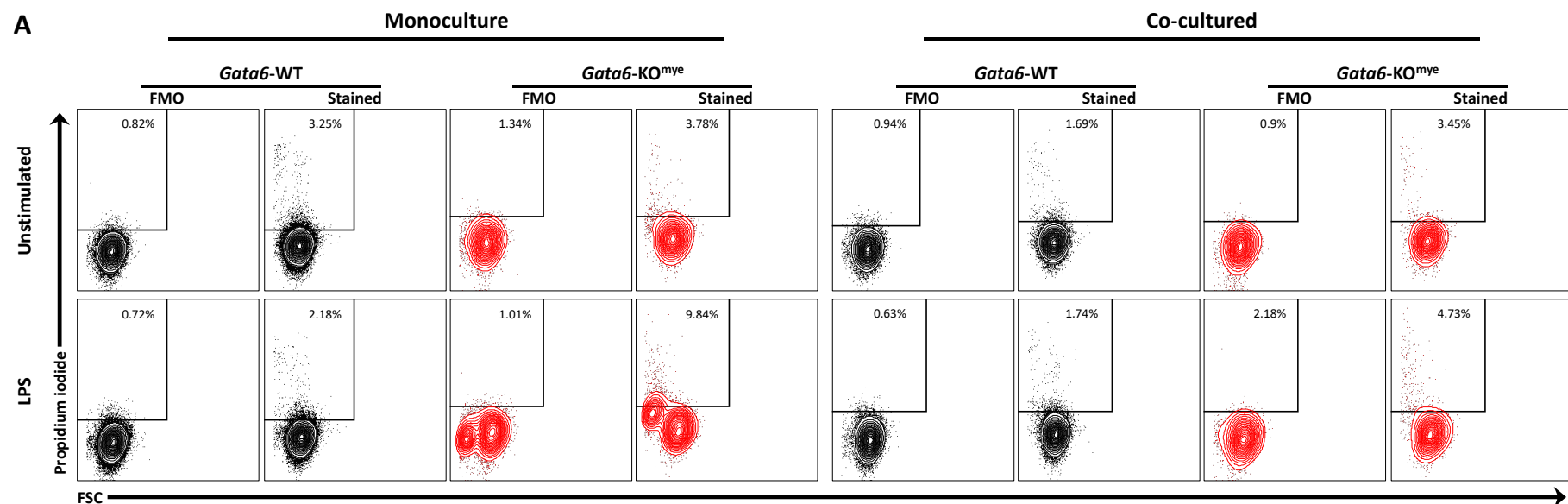


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Figure 3.14 | Cell-intrinsic release of IL-1 β from LPS-stimulated *Gata6*-deficient pM ϕ s is attenuated when co-cultured with pM ϕ s from *Gata6*-WT mice.

(A) Bivariate plot showing the uptake of propidium iodide (PI) in pM ϕ s (gated by Tim4⁺F4/80⁺) from *Gata6*-WT or *Gata6*-KO^{mye} mice that were cultured separately (monoculture) or co-cultured (1:1 total PECs) and either unstimulated or stimulated with 100 ng ml⁻¹ LPS for 16 hours. The percentage PI⁺ cells is indicated on each plot. Data representative of two independent experiments. (B) APECs from *Gata6*-WT and *Gata6*-KO^{mye} were cultured separately (1 x 10⁵ and 0.5 x 10⁵) or co-cultured at a 1:1 ratio (1 x 10⁵ total cells) after which they were stimulated with 100 ng ml⁻¹ LPS for 24 hours. After stimulation, IL-1 β release was determined in cell-free culture supernatants by ELISA. Data shows mean \pm S.D from four independent experiments. nd = not detectable. Statistical significance was determined by one-way ANOVA with Tukeys multiple comparison post-test between co-cultured cells and monocultures containing 0.5 x 10⁵ cells (*p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant).

3.3.15 Soluble Factors

Interestingly, a distinguishing difference between LPS and Pam3CSK4 was the capacity to induce the production of TNF. The secretion of TNF from LPS-stimulated *Gata6*-deficient pMφs was significantly higher than pMφs from *Gata6*-WT mice ($p < 0.0001$). Conversely, the secretion of TNF from *Gata6*-deficient pMφs stimulated with Pam3CSK4 was comparable to secretion from *Gata6*-WT mice ($p = 0.0773$) (Figure 3.16). TNF signalling can induce the activation of NF-κB as well as pro-apoptotic signalling cascades in macrophages (Parameswaran and Patial, 2010). Therefore, the differential IL-1β secretion and cell death in *Gata6*-deficient pMφs may be mediated by TNF produced by LPS-stimulated but not Pam3CSK4.

To address this, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice were unstimulated or stimulated with LPS in the presence or absence of etanercept, which functions as a decoy receptor thereby inhibiting TNF signalling (Figure 3.15). Additionally, pMφs were stimulated with LPS with or without recombinant IL-1 receptor antagonist (rIL-1Ra) to determine whether autocrine or paracrine IL-1 signalling exacerbates IL-1β production.

The secretion of IL-1β from LPS-stimulated *Gata6*-deficient pMφs treated with etanercept was reduced by over 30% compared to LPS stimulation alone ($p = 0.0049$). However, the release of IL-1β from *Gata6*-deficient pMφs stimulated with recombinant TNF was comparable to unstimulated pMφs ($p > 0.9999$) (Figure 3.15 C). The secretion of IL-1β from LPS-stimulated *Gata6*-deficient pMφs treated with recombinant IL-1RA was comparable to LPS stimulation alone ($p = 0.0214$).

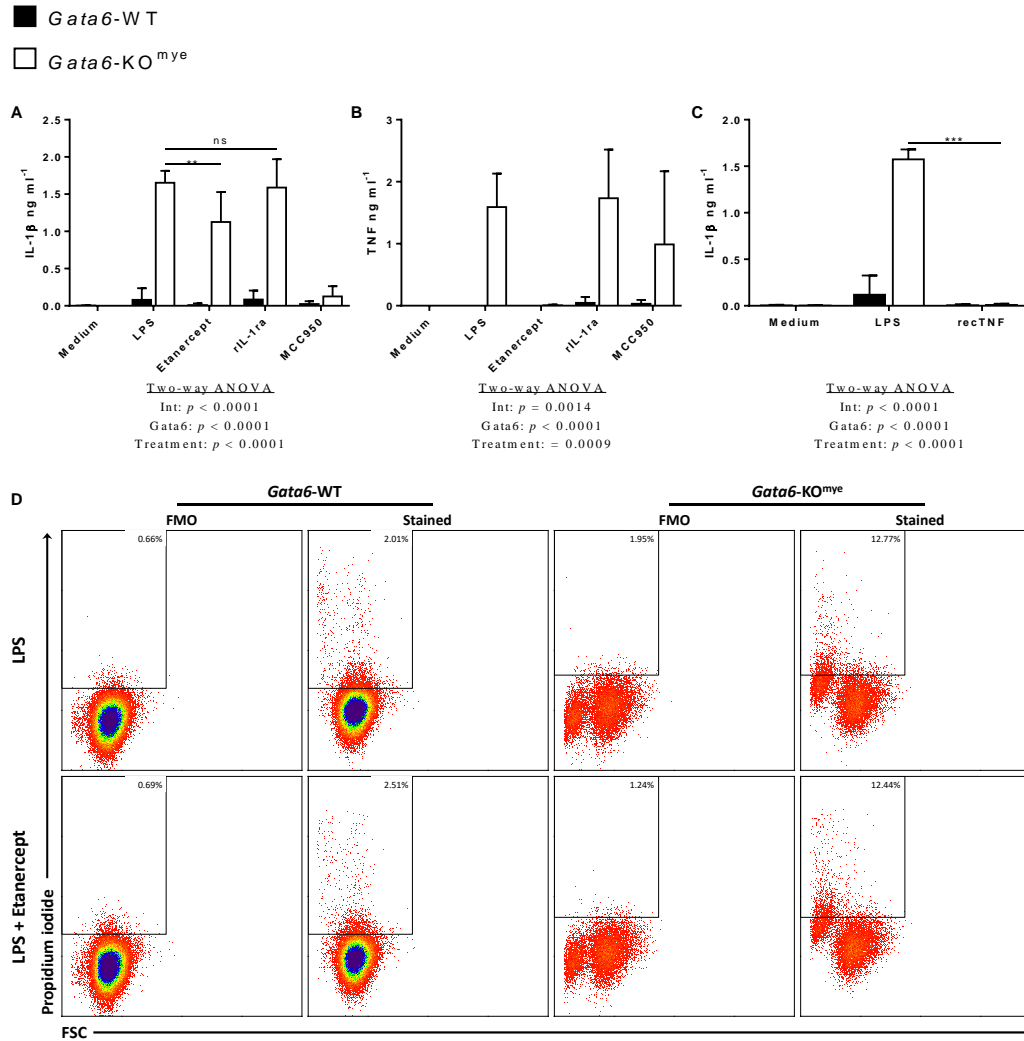


Figure 3.15 | IL-1β release is reduced from LPS-stimulated *Gata6*-deficient pMφs when TNF signalling is inhibited.

APECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were unstimulated or stimulated with 100 ng ml⁻¹ LPS in the presence or absence of 25 μg ml⁻¹ etanercept, 2.5 μg ml⁻¹ recombinant mIL-1RA, or 1 μM MCC950 for 24 hours. Culture supernatants were collected and (A) IL-1β and (B) TNF release measured by ELISA. Data are expressed as mean ± S.D from five independent experiments. (C) APECs were unstimulated or stimulated with either 100 ng ml⁻¹ LPS or 100 ng ml⁻¹ recombinant murine TNF (recTNF) for 24 hours, after which the supernatant was collected and IL-1β release measured by ELISA. Data are expressed as mean ± S.D from three independent experiments. (D) Bivariate plot showing the uptake of propidium iodide (PI) in pMφs (gated by Tim4⁺F4/80⁺) from *Gata6*-WT or *Gata6*-KO^{mye} mice that were stimulated with 100 ng ml⁻¹ LPS in the presence or absence of 25 μg ml⁻¹ Etanercept for 16 hours. The percentage PI⁺ cells is indicated on each plot. Experiment is representative of two independent experiments. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Gata6, Gata6 effects; Treatment, Treatment effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

3.3.16 Purinogenic signalling

Our group has previously reported that CD73 expression is greatly diminished in *Gata6*-deficient pMφs (Rosas et al., 2014), which may alter purinogenic signalling and consequently the production of pro-inflammatory cytokines. Adenosine generated through the processing of extracellular ATP by CD39 and CD73 is reported to mediate the down-regulation of pro-inflammatory cytokines and induce the production of IL-10 (Cohen et al., 2013, Hamidzadeh and Mosser, 2016).

To examine whether *Gata6*-deficient pMφ produce less IL-10 than those from *Gata6*-WT mice, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were either unstimulated or stimulated with either LPS or Pam3CSK4 and the production of IL-10 determined by ELISA (Figure 3.16). The release of IL-10 was significantly lower in pMφs from *Gata6*-KO^{mye} stimulated with LPS compared to those from *Gata6*-WT mice ($p = 0.0101$). Conversely, IL-10 production of pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were comparable ($p = 0.0533$).

To examine whether dysregulated purinogenic signalling is responsible for the release of IL-1β from *Gata6*-deficient pMφs upon LPS stimulation, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice were unstimulated or stimulated with LPS in the presence or absence of apyrase or adenosine and the release of IL-1β determined (Figure 3.16). The release of IL-1β from *Gata6*-deficient pMφs was not significantly reduced by treatment with apyrase (which hydrolyses extracellular ATP) compared to LPS-stimulation alone ($p = 0.7090$). Similarly, treatment with adenosine had no discernible effect on the release of IL-1β from *Gata6*-deficient pMφs stimulated with LPS alone ($p = 0.9816$). Furthermore, the secretion of IL-1β from LPS-stimulated pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice treated with adenosine 5'-(α,β-methylene) diphosphate (AMP-CP), a CD73 inhibitor, was comparable to LPS-stimulation alone ($p = 0.9858$ and $p = 0.8468$ respectively).

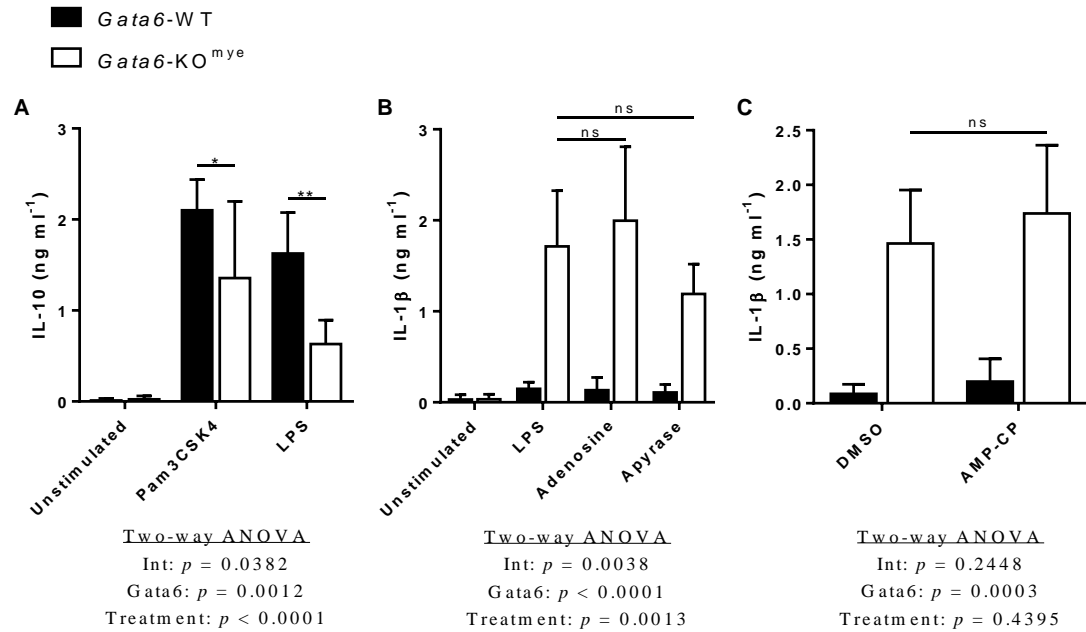


Figure 3.16 | LPS-stimulated *Gata6*-deficient pM ϕ s secrete less IL-10 but IL-1 β release is unaffected by purinogenic signalling.

(A) APECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were unstimulated or stimulated with either 500 ng ml⁻¹ Pam3CSK4 or 100 ng ml⁻¹ LPS for 24 hours. Culture supernatants were collected and IL-10 release measured by ELISA. Data are expressed as mean \pm S.D from five independent experiments. (B, C) APECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were unstimulated or stimulated with 100 ng ml⁻¹ LPS in the presence or absence of 20 U ml⁻¹ apyrase, 100 μ M adenosine, or 10 μ M AMP-CP for 24 hours. Culture supernatants were collected and IL-1 β release measured by ELISA. Data are expressed as mean \pm S.D from three independent experiments. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Gata6, Gata6 effects; Treatment, Treatment effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

3.3.17 Loss of *Gata6* may enhance neutrophil recruitment *in vivo*

IL-1 β has a key function in the recruitment of neutrophils, and the IL-1-dependent peritoneal accumulation of neutrophils has frequently been used as an *in vivo* assay for IL-1 β production (Martinon et al., 2006, Guarda et al., 2009, Duewell et al., 2010). Furthermore, macrophages secrete a plethora of soluble factors that can drive neutrophil recruitment including cytokines such as CCL3, CXCL2, and CXCL1 (Sadik et al., 2011).

To determine whether *Gata6*-deficient pM ϕ s would drive a significant increase in neutrophil recruitment and/or retention, *Gata6*-WT and *Gata6*-KO^{mye} were injected with either 1 ng or 10 ng LPS for 4 hours and the number of neutrophils determined by flow cytometry (Figure 3.17 A). The number of neutrophils recruited to the peritoneal cavity of *Gata6*-KO^{mye} was significantly higher than *Gata6*-WT mice injected with 1 ng LPS ($p = 0.0161$). Conversely, mice injected with 10 ng LPS had comparable numbers of neutrophils recruited into the peritoneal cavity of *Gata6*-WT and *Gata6*-KO^{mye} ($p = 0.1534$). To determine what cytokines and chemokines might drive the differential influx of neutrophils, peritoneal lavage supernatants from mice injected with 1 ng and 10 ng LPS were analysed by ELISA (Figure 3.17 B). However, no significant differences in cytokine and chemokine production were observed between *Gata6*-WT and *Gata6*-KO^{mye}, and many of the cytokines and chemokines analysed were non-detectable or very low. To further investigate what factors might enhance the recruitment of neutrophils *in vivo*, pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} stimulated with LPS *ex vivo* for 3 hours and the production of chemokines determined by ELISA (Figure 3.17 D). The production of CXCL1 and CXCL2 from LPS-stimulated *Gata6*-deficient pM ϕ s is comparable to those from *Gata6*-WT mice ($p = 0.065$ and $p = 0.477$ respectively). Conversely, the production of CCL3 was significantly higher in pM ϕ s from *Gata6*-KO^{mye} stimulated with LPS higher compared to *Gata6*-WT pM ϕ s ($p = 0.0006$).

To establish whether *Gata6*-KO^{mye} had increased retention or recruitment of neutrophils at later time points, mice were injected with LPS and the recruitment of neutrophils determined by flow cytometry. Unlike at 4 hours, there was no significant difference in the number of neutrophils recruited after 24 or 48 hours into the

peritoneal cavities of *Gata6*-WT and *Gata6*-KO^{mye} mice following the intraperitoneal injection of 1 ng LPS ($p = 0.9922$ and $p = 0.8005$ respectively).

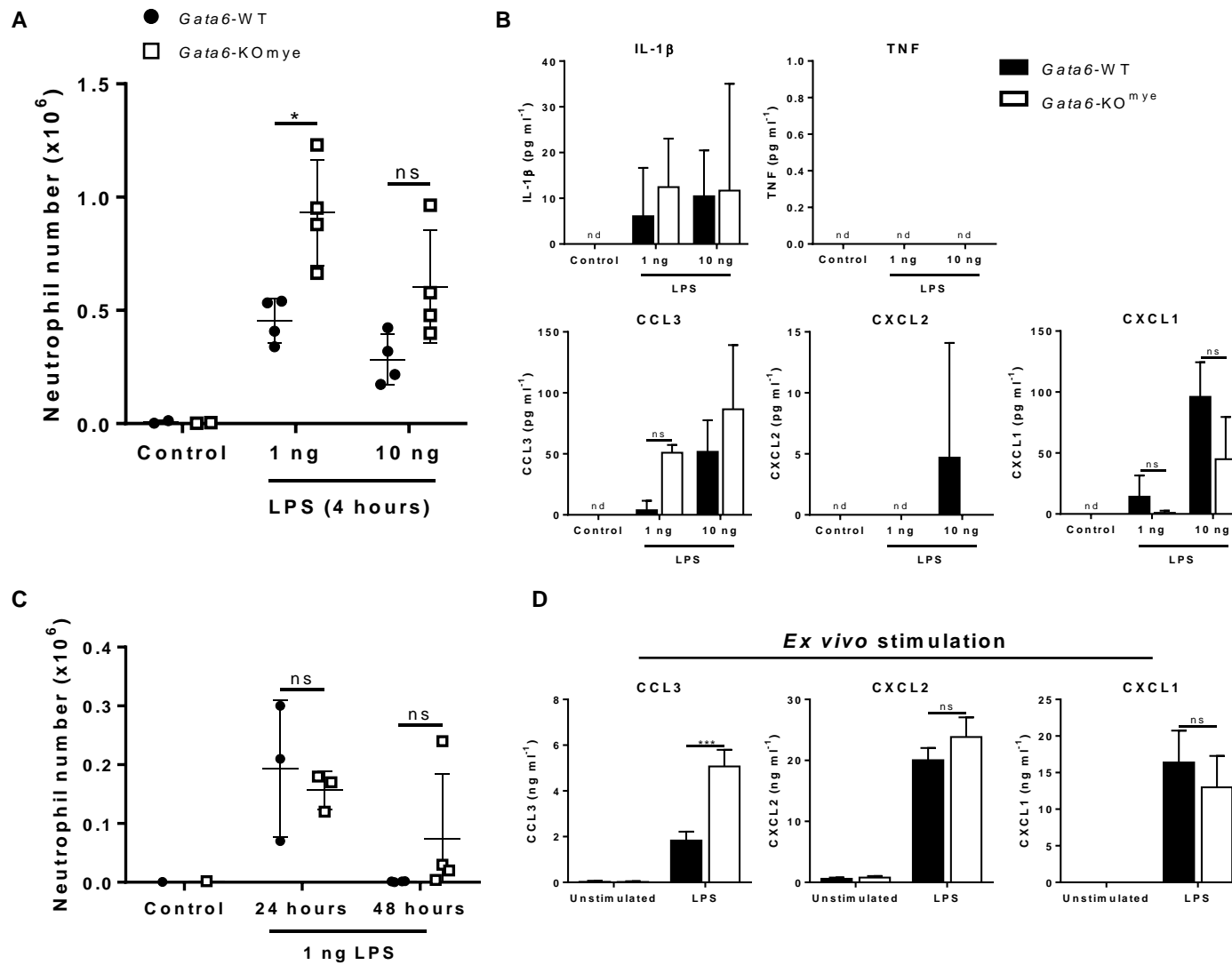


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Figure 3.17 | *Gata6*-deficiency may enhance neutrophil influx.

(A) *Gata6*-WT and *Gata6*-KO^{mye} mice were either not injected (control) or injected intraperitoneally with either 1 ng or 10 ng LPS. Peritoneal lavage cells were analysed by flow cytometry for neutrophil recruitment after 4 hours. Scatter plot shows the number of neutrophils with each symbol representing one mouse. (B) Bar charts show cytokine and chemokine in peritoneal lavage supernatant from (A) was determined by ELISA. nd = not detectable. (C) *Gata6*-WT and *Gata6*-KO^{mye} mice were either not injected (control) or injected intraperitoneally with 1 ng LPS. Peritoneal lavage cells were analysed by flow cytometry for neutrophil recruitment after 24 or 48 hours. Scatter plot shows the number of neutrophils with each symbol representing one mouse. (D) APECs from *Gata6*-WT and *Gata6*-KO^{mye} were unstimulated or stimulated with 100 ng ml⁻¹ LPS for 3 hours. Culture supernatants were collected 3 hours after stimulation and CCL3, CXCL2, and CXCL1 release measured by ELISA. Data are shown as mean ± S.D from five independent experiments. Statistical significance was determined by two-way ANOVA with Tukeys multiple comparison post-test (*p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant). (A, C) Injections and flow cytometry were performed by Dr Natacha Ipseiz. I assisted with peritoneal lavages and cell counting, experimental design, and measuring cytokines and chemokines in peritoneal lavage supernatants by ELISA.

3.4 Discussion

The aim of this chapter was to investigate whether *Gata6* controls the inflammatory response in peritoneal macrophages.

3.4.1 LPS-induces IL-1 β and IL-18 secretion

The production of IL-1 β is tightly-regulated and in macrophages, requires at least two distinct signals. The first signal (typically TLR ligands) induces the expression of pro-IL-1 β , which is biologically inactive. The second signal is required for the activation of caspase-1, which cleaves pro-IL-1 β (and IL-18) into the biologically active mature forms, which then exit the cell and exert their biological effects through their cognate receptors. The activation of caspase-1 is generally mediated through multimeric protein complexes termed inflammasomes, of which the NLRP3 inflammasome is the best characterised.

Strikingly, *Gata6*-deficient pM ϕ s stimulated with LPS alone secreted mature IL-1 β and IL-18, whereas pM ϕ s from *Gata6*-WT mice did not secrete IL-1 β or IL-18 (Figure 3.1). Although the release of mature IL-1 β in response to LPS alone ('Priming' signal) has been well described for murine BMDCs and human monocytes (Gaidt et al., 2016, He et al., 2013), secretion from macrophages is generally strictly dependent on two distinct signals. Conversely, genetic defects that lead to spontaneous IL-1 β secretion by macrophages have been reported. BMDMs deficient of all three inhibitor of apoptosis proteins (cIAP1, cIAP2, and XIAP) exhibited spontaneous NLRP3 inflammasome activity and IL-1 β secretion in response to LPS alone (Vince et al., 2012). Similarly, ablation of *Atg16l1* resulted in IL-1 β secretion in response to LPS stimulation alone (Saitoh et al., 2008). More recently, LPS-stimulated BMDMs deficient of *Tnfrsf3* (which encodes the NF- κ B inhibitor A20) were shown to spontaneously secrete IL-1 β (Duong et al., 2015). However, the molecular mechanisms governing the secretion of IL-1 β from LPS-stimulated *Gata6*-deficient pM ϕ s is likely to be distinct from these reported studies. For example, *Gata6*-deficient pM ϕ s primed with LPS and stimulated with soluble NLRP3 stimuli (ATP and nigericin) responded normally, secreting comparable levels of IL-1 β as pM ϕ s from *Gata6*-WT mice (Figure 3.5). Conversely, macrophages deficient of all IAP proteins,

A20 or Atg16L1 primed with LPS and activated with ATP secreted significantly more IL-1 β compared to wild-type macrophages. Another distinguishing feature is the specificity of LPS to induce the secretion of IL-1 β , but not any of the other TLR ligands tested, whereas macrophages deficient of all IAP proteins or A20 secreted mature IL-1 β in response to a range of TLR ligands.

3.4.2 IL-1 β release is restricted to LPS stimulation

Having established that LPS-stimulated *Gata6*-deficient pM ϕ s secreted mature IL-1 β , I investigated whether stimulation with other TLR ligands alone would induce the release of mature IL-1 β . Somewhat surprisingly, none of the other TLR ligands tested induced a significant release of IL-1 β from *Gata6*-deficient pM ϕ s (Figure 3.2). To investigate the mechanisms governing the differential release of IL-1 β in response to different TLR ligands, pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} mice were stimulated with LPS or Pam3CSK4 and the expression of pro-IL-1 β determined. The expression of pro-IL-1 β was comparable between LPS- and Pam3CSK4-stimulated *Gata6*-deficient pM ϕ s, indicating that Pam3CSK4 was an effective priming stimulus. Supporting this, studies have shown that pM ϕ s primed with Pam3CSK4 and subsequently treated with ATP release mature IL-1 β (Levesque et al., 2010). Thus, Pam3CSK4 stimulation induces the expression of pro-IL-1 β and NLRP3 expression, but is ordinarily incapable of inducing the release of mature IL-1 β , consistent with the two-signal hypothesis. Based on these observations, the release of IL-1 β from LPS-stimulated but not Pam3CSK4-stimulated *Gata6*-deficient pM ϕ s suggest that downstream molecular mechanisms are differentially induced by LPS and Pam3CSK4 which mediate the activation of the inflammasome. However, for the studies carried out in this thesis, a dose-response curve for IL-1 β and IL-18 release from *Gata6*-WT and *Gata6*-KO^{mye} pM ϕ s was only performed with the TLR4 ligand LPS. As such, it is possible that the concentrations of TLR ligands used to stimulate pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} pM ϕ s were sub-optimal. As such, it is possible that the release of IL-1 β and IL-18 from *Gata6*-deficient pM ϕ s may not be restricted to LPS stimulation alone and that the failure of other TLR ligands to induce the release of IL-1 β and TNF from *Gata6*-KO^{mye} is an experimental artefact.

3.4.3 Mitochondrial dysfunction

Having established that the secretion of IL-1 β from LPS-stimulated *Gata6*-deficient pM ϕ s was mature, the underlying molecular mechanisms of NLRP3 inflammasome activation were investigated. Given the structural and molecularly diverse array of NLRP3 activatory stimuli, it is unlikely that they all bind directly to the NLRP3 inflammasome, as such, it has been suggested that inflammasomes most likely monitor cellular homeostasis which is perturbed by activatory stimuli. In addition to their role in metabolism, mitochondria are complex regulators of cellular homeostasis. Perturbation of ROS, intracellular K⁺ concentration or lysosomal damage have been reported to induce mitochondrial dysfunction (Tschopp, 2011), which are also implicated in the activation of the NLRP3 inflammasome. As such, mitochondrial dysfunction has been strongly linked to the activation of the NLRP3 inflammasome, predominantly through the generation of mtROS and the release of mtDNA and cardiolipin (Nakahira et al., 2011, Zhou et al., 2011, Iyer et al., 2013). However, the role of mitochondria in the activation of the NLRP3 inflammasome is contentious (Lawlor and Vince, 2014). Naïve *Gata6*-deficient pM ϕ s had enhanced mtROS compared to pM ϕ s from *Gata6*-WT mice. Consistent with increased mtROS, *Gata6*-deficient pM ϕ s had a higher basal rate of OXPHOS than pM ϕ s from *Gata6*-WT mice, although the spare capacity of *Gata6*-deficient pM ϕ s was lower. Furthermore, both unstimulated and LPS-stimulated *Gata6*-deficient pM ϕ s had significantly more dysfunctional mitochondria than pM ϕ s from *Gata6*-WT, as determined by MitoTracker deep red (selective for respiring mitochondria) staining relative to MitoTracker Green (Figure 3.7).

3.4.4 *Gata6* restricts LPS-induced cell death

In addition to the cleavage of IL-1 β and IL-18, caspase-1 mediates pyroptotic cell death in macrophages. To investigate whether LPS-stimulation induces cell death in *Gata6*-deficient pM ϕ s, unstimulated and LPS-stimulated pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} mice were analysed by flow cytometry for propidium iodide (PI⁺), a membrane impermeant dye that is generally excluded from viable cells. *Gata6*-deficient pM ϕ s stimulated with LPS had a significant increase in the number of PI⁺ pM ϕ s, whereas unstimulated *Gata6*-deficient pM ϕ s and both unstimulated and LPS-

stimulated pMφs from *Gata6*-WT mice had comparable numbers of PI⁺ cells (Figure 3.8). Furthermore, the PI⁺ pMφs exhibited a marked decrease in forward scatter profile, indicative of dead cells. Interestingly, the number of PI⁺ *Gata6*-deficient pMφs stimulated with Pam3CSK4 was comparable to unstimulated pMφs and both unstimulated and Pam3CSK4-stimulated pMφs from *Gata6*-WT mice (Figure 3.13). Thus, the restriction of cell death and IL-1β secretion to LPS stimulation but not Pam3CSK4 stimulation indicates that the mechanisms governing the secretion of IL-1β in LPS-stimulated *Gata6*-deficient pMφs also mediated cell death.

Inhibition of caspase-1 and NLRP3 activation resulted in a significant reduction of IL-1β secretion from LPS-stimulated pMφs, consistent with a requirement of NLRP3 mediated activation of caspase-1 and the processing of pro-IL-1β by caspase-1 to the mature biologically active form (Figure 3.4). However, inhibition of caspase-1 or NLRP3 activation failed to inhibit LPS-induced cell death in *Gata6*-deficient pMφs (Figure 3.9). The failure of caspase-1 and NLRP3 inhibition to prevent LPS-induced cell death was unexpected, as caspase-1-dependent cleavage of gasdermin d was recently shown to mediate pyroptotic cell death (He et al., 2015, Kayagaki et al., 2015, Shi et al., 2015). Alternative mechanisms of caspase-1-independent pyroptosis have been shown to be mediated by caspase-11, which detects intracellular LPS (Kayagaki et al., 2015). After activation, caspase-11 directly mediates pyroptosis and activation of the NLRP3 inflammasome. The NLRP3 inflammasome subsequently leads to the activation of caspase-1, which cleaves pro-IL-1β. As such, further study is needed to investigate whether the cell death is mediated through the cleavage of gasdermin d, or whether the induction of cell death by LPS is independent from the molecular events regulating the release of IL-1β.

Alternatively, LPS-stimulation might induce another mode of cell death in *Gata6*-deficient pMφs. The marked reduction pMφs in *Gata6*-KO^{mye} was recently attributed to a significant increase apoptosis of naïve *Gata6*-deficient pMφs (Gautier et al., 2014), although an alternative explanation for reduced numbers was provided by another study indicating that *Gata6*-deficient pMφs are retained in the omentum (Okabe and Medzhitov, 2014). In contrast to the observations of Gautier and colleagues, there was no significant difference in the percentage of caspase-3⁺ pMφs freshly isolated from *Gata6*-WT and *Gata6*-KO^{mye} mice (Figure 3.10). Similarly, annexin V staining was comparable between naïve pMφs from *Gata6*-WT and *Gata6*-KO^{mye}, which both

exhibited a comparable shift in staining compared to FMO controls. The background staining of annexin V can be attributed to the constitutive cell surface expression of Annexin V binding phospholipids in macrophages (Callahan et al., 2000). The increase in apoptosis of *Gata6*-deficient pMφs was attributed to the marked reduction in *Aspa* expression, although it is noteworthy that approximately 10% of pMφs from *Aspa*-deficient mice were apoptotic, compared to over 20% reported for *Gata6*-deficient pMφs (Gautier et al., 2014). Furthermore, apoptotic cells *in vivo* are typically cleared rapidly, unless the phagocytes have an impairment in the phagocytosis of apoptotic cells (Taylor et al., 2000, Elliott and Ravichandran, 2016, O'Brien et al., 2006). Conversely, LPS stimulation induced a significant increase in the percentage of caspase-3⁺ pMφs from *Gata6*-KO^{mye} compared to unstimulated pMφs and both unstimulated and LPS-stimulated pMφs from *Gata6*-WT mice (Figure 3.11).

Interestingly, the percentage of PI⁺ pMφs was lower in *Gata6*-deficient pMφs co-cultured with those from *Gata6*-WT mice (Figure 3.14). Furthermore, the release of IL-1β from LPS-stimulated co-cultured cells was significantly reduced compared to monocultures of *Gata6*-deficient pMφs (Figure 3.14). This suggests that *Gata6* may regulate cell-contact dependent mechanisms or release of soluble factors that modulate cell death and inflammatory responses.

3.4.5 IL-1β secretion is enhanced by TNF signalling, but not strictly dependent

Having established that co-cultured pMφs release significantly less IL-1β than monocultures of *Gata6*-deficient pMφs, I sought to establish whether soluble factors released from *Gata6*-WT pMφs may attenuate the aberrant inflammatory responses of *Gata6*-deficient pMφs. *Gata6*-deficient pMφs stimulated with LPS had significantly higher and prolonged production of TNF compared to pMφs from *Gata6*-WT mice. Intriguingly, the production of TNF was comparable between pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice stimulated with Pam3CSK4, which was undetectable at 24 hours (Figure 4.4). TNF signalling can induce the activation of NF-κB as well as pro-apoptotic signalling cascades in macrophages. Therefore, the differential IL-1β secretion and cell death in *Gata6*-deficient pMφs may be mediated by TNF produced by LPS-stimulated but not Pam3CSK4. Blocking TNF signalling with Etanercept

significantly reduced the secretion of IL-1 β from LPS-stimulated *Gata6*-deficient pM ϕ s by approximately 30%. Conversely, the production of IL-1 β from *Gata6*-deficient pM ϕ s stimulated with recombinant TNF was comparable to unstimulated pM ϕ s and both unstimulated and TNF-stimulated pM ϕ s from *Gata6*-WT mice. TNF may therefore synergise with LPS and induce the secretion of IL-1 β from *Gata6*-deficient pM ϕ s. In contrast to the reduction of IL-1 β secretion, the percentage of PI⁺ pM ϕ s from *Gata6*-KO^{mye} mice stimulated with LPS alone was comparable to those treated with etanercept (Figure 3.15), suggesting that TNF signalling does not play a prominent role in the cell death of LPS-stimulated *Gata6*-deficient pM ϕ s. Overall, these findings suggest that TNF may exacerbate the production of IL-1 β but does not mediate cell death following LPS treatment. Blocking IL-1 signalling with IL-1RA had no discernible effect on the release of IL-1 β .

In addition to TNF, the production of IL-10 was significantly down-regulated in LPS-stimulated *Gata6*-deficient pM ϕ s compared to those from *Gata6*-WT mice (Figure 3.16). Interestingly, adenosine signalling can down-regulate the expression of pro-inflammatory cytokines including TNF and increase the expression of anti-inflammatory cytokines such as IL-10. Our group previously reported that CD73 was significantly downregulated in *Gata6*-deficient pM ϕ s (Rosas et al., 2014), an ectoenzyme that catalyses ADP to adenosine. Upon stimulation with TLR ligands, macrophages release ATP into the extracellular space, where CD39 converts ATP to ADP, which is subsequently dephosphorylated by CD73 to adenosine. These ectoenzymes thereby mediate inflammatory signalling in two ways: the removal of ATP from the extracellular space which can otherwise activate the NLRP3 inflammasome, and the generation of adenosine, which upon binding to its receptor induces the expression of IL-10 and the down-regulation of pro-inflammatory cytokines including TNF. The release of IL-1 β was comparable between *Gata6*-deficient pM ϕ s stimulated with LPS alone and in the presence of adenosine (Figure 3.16). Supporting this, inhibition of CD73 in pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} did not significantly affect the release of IL-1 β from LPS stimulation alone, suggesting that adenosine generated through CD73 does not have a prominent role regulating the release of IL-1 β . Treatment with extracellular apyrase (CD39) did not significantly reduce IL-1 β release from LPS-stimulated *Gata6*-deficient pM ϕ s, suggesting that

extracellular ATP does not activate the NLRP3 inflammasome and subsequent release of IL-1 β .

3.4.6 *Gata6* may restrict neutrophil recruitment and retention

One of the major functions of IL-1 β *in vivo* is the recruitment of neutrophils (Martinon et al., 2006, Duewell et al., 2010). Since *ex vivo* LPS-stimulated *Gata6*-deficient pM ϕ s secrete significant amounts of mature IL-1 β , attention was directed to determine whether *Gata6*-deficiency would enhance the recruitment and/or retention of neutrophils *in vivo* after administration of LPS intraperitoneally. *Gata6*-KO^{mye} exhibited a significant increase in the number of neutrophils in the peritoneal cavity 4 hours after intraperitoneal injection of LPS compared to *Gata6*-WT mice, although these results are from one independent experiment, and thus further repeats are needed to establish whether *Gata6*-deficient does promote enhanced neutrophil recruitment and retention (Figure 3.17). *Gata6*-deficient pM ϕ s stimulated with LPS *ex vivo* secreted detectable levels of IL-1 β around 12 hours, so it is currently unclear whether IL-1 β secretion mediates the significant increase in neutrophils 4 hours post LPS administration. On the other hand, LPS injected *in vivo* may induce the production of cytokines, chemokines, and lipid mediators with different kinetics to those of *ex vivo* stimulated pM ϕ s. IL-1 β signalling has been shown to drive the influx of neutrophils through the secondary production of CXCL1 and CXCL2 (Biondo et al., 2014). In contrast to earlier time points, there was no significant difference in the number of neutrophils in the peritoneal cavities of *Gata6*-WT and *Gata6*-KO^{mye} mice following intraperitoneal administration of LPS for 24 hours. At 48 hours post LPS administration, no neutrophils were detectable in the peritoneal cavities of *Gata6*-WT mice, whereas some neutrophils were detected in the cavities of *Gata6*-KO^{mye} mice, albeit in low numbers (Figure 3.17). Peritoneal lavage supernatant was collected after 4 hours and the production of cytokines and chemokines measured by ELISA. However, no significant differences were observed between *Gata6*-WT and *Gata6*-KO^{mye}, and many of the cytokines and chemokines analysed were non-detectable or very low. The limited detection of cytokines and chemokines may reflect the time point in which peritoneal lavage supernatant was collected. One study looking at the production of chemokines in the peritoneal cavity following challenge with *Staphylococcus epidermidis* supernatant showed that chemokines are produced transiently and not detectable after 3 hours (CXCL1 and CXCL2), or detectable in very small quantities (CCL3) during the time points analysed (Perks et al., 2016). However,

ex vivo stimulated *Gata6*-deficient pMφs secreted significantly more CCL3 than those from *Gata6*-WT mice, which could drive neutrophil influx. However, CCL3 production is comparatively much lower than both CXCL1 and CXCL2, which are reported to be the major chemoattractant responsible for attracting neutrophil migration (De Filippo et al., 2013, Ritzman et al., 2010). Therefore, the relative contribution of increased CCL3 driving a significant increase in neutrophil migration into the peritoneum remains to be investigated. Interestingly, *Gata6*-deficient pMφs stimulated *ex vivo* with LPS for 16 hours had sustained production of CCL3 compared to pMφs from *Gata6*-WT mice.

3.4.7 Conclusions

Collectively, these results suggest that *Gata6* exerts critical control, either directly or indirectly, on the control of peritoneal macrophage inflammatory responses. Specifically, *Gata6*-deficient pMφs have perturbed inflammatory responses where they secrete mature IL-1 β in response to LPS alone. Ordinarily, macrophages require two-signals for the release of mature IL-1 β , so these observations are particularly striking.

Chapter Four

The role of prostacyclin/prostacyclin synthase in peritoneal macrophage function

4.1 Introduction

Soluble mediators in inflammation

Soluble mediators play a critical role in the acute inflammatory response, and are released by both immune and non-immune cells upon pathogen recognition. When released, they orchestrate a wide array of functions such as the recruitment of effector cells, the polarisation/priming of both resident and infiltrating cells to enhance pathogen killing and direct antimicrobial activity. Furthermore, soluble mediators are critical for the resolution of inflammation, promoting tissue repair and ultimately the restoration of tissue homeostasis. Therefore, the spatiotemporal release of a diverse array of soluble mediators must be tightly regulated. Lipid mediators are potent signalling molecules that regulate an array of cellular responses, including in inflammation and immunity. Furthermore, dysregulated lipid signalling has been implicated in an array of inflammatory diseases (Ricciotti and FitzGerald, 2011). Supporting this, dysregulated control of soluble mediators is a major contributing factor to the onset and maintenance of several inflammatory and autoimmune diseases such as rheumatoid arthritis, peritonitis, diabetes, and inflammatory bowel disease (Funk, 2001).

Tissue-specific

Studies in our lab showed that *Gata6*-deficient pMφs had perturbed inflammatory responses (data unpublished), suggesting that *Gata6* may regulate, directly or indirectly, the expression of genes in pMφs involved in host defence and inflammation. Microarray analysis of pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice revealed that a large number of pMφ-specific genes were downregulated in *Gata6*-deficient pMφs (Rosas et al., 2014, Gautier et al., 2014). Interestingly, one of the most down-regulated genes identified in the array was prostacyclin synthase (*Ptgis*), which was over 16-fold down-regulated in *Gata6*-deficient pMφs. *Ptgis* is a key enzyme required for the synthesis of prostacyclin (PGI₂), a member of the eicosanoid family.

Prostacyclin (PGI₂) expression in homeostasis and disease

The eicosanoids comprise a large family of bioactive lipids derived from arachidonic acid, which have diverse roles in regulating homeostatic processes and in regulating inflammation and immune responses (Funk, 2001). Macrophages are important sources of eicosanoids, which are produced rapidly in a multi-step process upon recognition of bacterial and fungal ligands (Peters-Golden et al., 2005, Alvarez et al., 2010, Norris et al., 2011, Suram et al., 2013). Upon stimulation, arachidonic acid from phospholipids are released into the intracellular space, which is subsequently metabolised through the 5-lipoxygenase pathway to leukotrienes and the COX pathway to prostanoids (Figure 4.1).

PGI₂ is perhaps best known for its role within the cardiovascular system as a potent vasodilator and inhibitor of platelet aggregation (anti-thrombotic). However, it has become increasingly evident that PGI₂ is important in the inflammatory responses. The key roles of prostanoids in inflammation were first demonstrated in 1971, in which the mechanism of action of aspirin was shown to be through the inhibition of prostanoid biosynthesis (Vane, 1971). Further work since has demonstrated both pro- and anti-inflammatory roles of different prostanoids, which are largely dependent on receptor expression and cell-type specific signalling pathways, reviewed by (Narumiya et al., 1999).

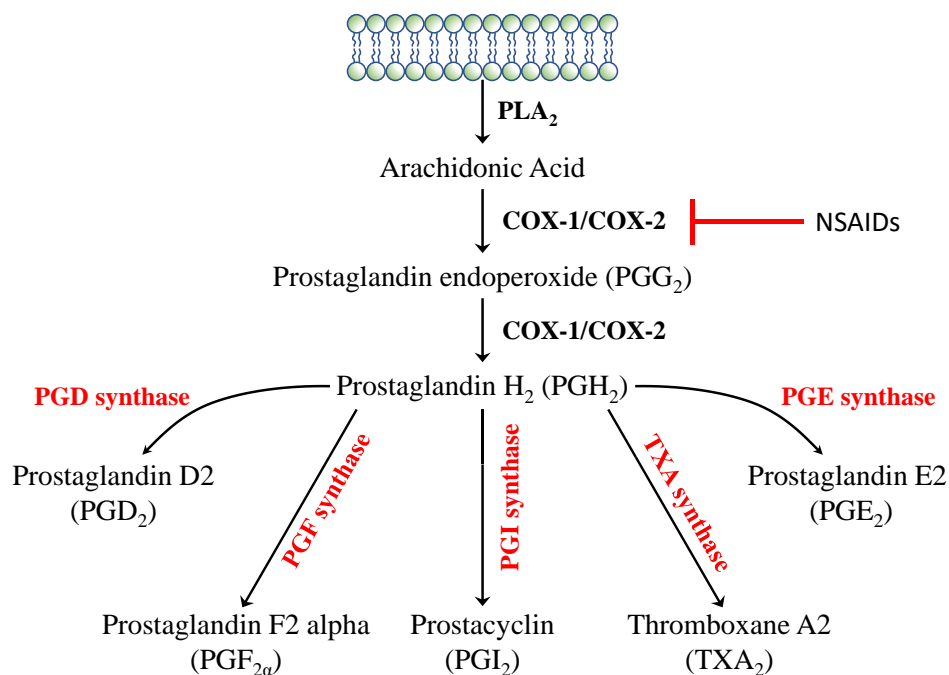


Figure 4.1 | Prostanoid synthesis pathway.

Prostanoids are synthesised from arachidonic acid, which is either liberated from the lipid membrane by PLA2 or taken up from the extracellular milieu. COX 1/COX 2 catalyse arachidonic acid into PGG2 and subsequently into prostaglandin H2 (PGH2), which is the common substrate for prostanoid synthesis. PGH2 is processed into individual prostanoids by the action of specific terminal synthases (red text). NSAIDs such as indomethacin inhibit the action of COX1/2 thereby preventing the production of downstream prostanoids.

Prostacyclin signalling

Once synthesised, PGI₂ rapidly diffuses across the plasma membrane and exerts its effects through binding to its cognate receptor, the prostacyclin receptor (IP receptor). The IP receptor is a GPCR that upon activation, initiates a signalling cascade culminating in the activation of adenylyl cyclase and PLC, which subsequently increase intracellular cAMP and decreases Ca²⁺ levels. These secondary signalling molecules exert a wide array of effects, which is cell-type specific and responsible for the biological actions of PGI₂. More recently, intracellular PGI₂ has also been reported to signal via nuclear peroxisome proliferator-activator receptors (PPARs), activating intracrine nuclear pathways (Figure 4.2).

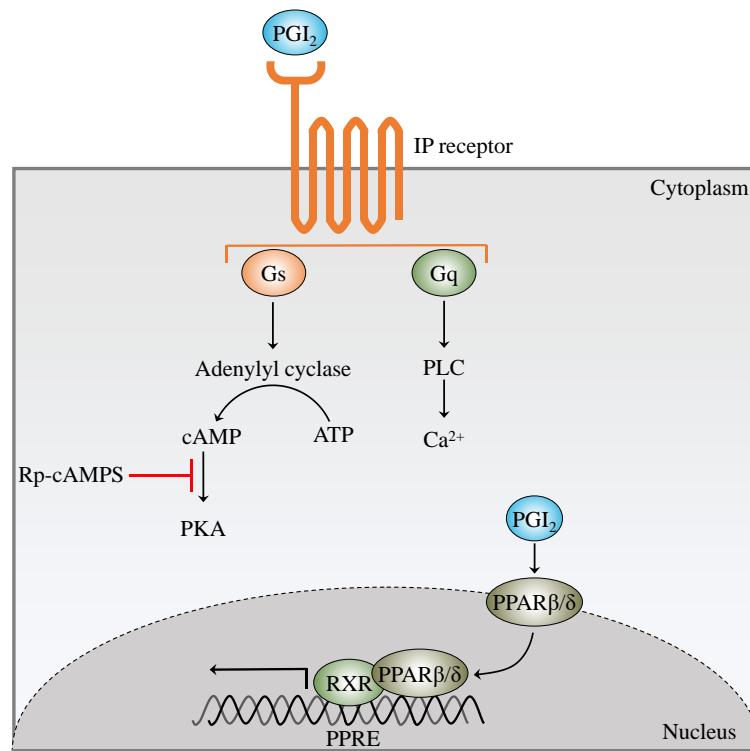


Figure 4.2 | Prostacyclin signalling: autocrine, paracrine, and intracrine mechanisms.

Autocrine and paracrine signalling by PGI₂ is mediated through the prostacyclin receptor (IP). Receptor binding predominantly activates Gs, though it can activate Gq. Gs activates adenylyl cyclase, which leads to increased cAMP. The cAMP mediates the activation protein kinase A (PKA). Intracrine PGI₂ signalling is mediated through PPARβ/δ, located on nuclear membranes. PPARs heterodimerise with RXRs in the presence and absence of ligand. After ligand binding, these heterodimers undergo conformational changes, resulting in the dissociation of co-repressors and the binding of coactivators. These heterodimers bind to DNA sequences called PPAR response elements (PPRE), leading to the transcription of target genes.

4.2 Chapter hypothesis and aims

The hypothesis tested in this chapter is that prostacyclin synthase plays a fundamental role in the control of peritoneal macrophage host defence and inflammation.

Aims

- To establish whether prostacyclin influences pro-inflammatory cytokine production.
- To determine the expression pattern of *Ptgis* across immune cell populations.
- Determine the expression of *Ptgis* in pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice
- To evaluate the impact of prostacyclin analogues on pro-inflammatory cytokines production in *Gata6*-deficient pMφs
- Design lentivirus construct expressing *Ptgis* transgene and evaluate whether it can modulate pro-inflammatory cytokine production in BMDMs
- Determine whether the *in vivo* reconstitution of *Ptgis* expression in *Gata6*-deficient pMφs restores pro-inflammatory cytokine production to pMφs from *Gata6*-WT mice
- To evaluate whether the *in vivo* knockdown of *Ptgis* expression in C57/BL6 mice recapitulates the proinflammatory cytokine production defects observed in *Gata6*-deficient pMφs

4.3 Results

4.3.1 LPS-stimulated *Gata6*-deficient pMφs have altered pro-inflammatory cytokine production

Preliminary studies in our lab showed that the loss of *Gata6* enhanced the production of pro-inflammatory cytokines, suggesting that tissue-specific transcription factors may mediate the acute inflammatory response and resolution of inflammation. TNF and IL-6 are the prototypical pro-inflammatory cytokines produced by pMφs upon recognition of microbial ligands and their dysregulated production has been implicated in a variety of human inflammatory diseases. To ascertain whether the loss of *Gata6* affected the production of these cytokines, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were either unstimulated or stimulated with LPS for 3 and 16 hours, after which the expression of *Tnf* and *Il6* were determined by qPCR (Figure 4.3). Furthermore, the kinetic release of TNF and IL-6 in response to LPS and zymosan was determined by ELISA (Figure 4.3).

The expression of both *Tnf* and *Il6* in unstimulated pMφs from *Gata6*-WT and *Gata6*-KO^{mye} was very low, in accordance of their typical requirement of induction by microbial ligands. However, stimulation with LPS for 3 hours resulted in greatly increased *Tnf* and *Il6* expression, which were both higher in pMφs from *Gata6*-KO^{mye} mice compared to *Gata6*-WT mice (Figure 4.3). After 16 hours, *Tnf* expression was undetectable in pMφs from *Gata6*-WT mice. Conversely, *Tnf* expression was higher in pMφs from *Gata6*-KO^{mye} mice compared to *Gata6*-WT mice (Figure 4.3). Remarkably, the expression of *Tnf* in *Gata6*-deficient pMφ stimulated with LPS for 16 hours was comparable to peak *Tnf* expression in pMφs from *Gata6*-WT mice. Similarly, the expression of *Il6* was greatly increased in *Gata6*-deficient pMφs compared to pMφs from *Gata6*-WT mice.

Having established that *Gata6*-deficient pMφs have enhanced transcription of pro-inflammatory genes in response to LPS stimulation, the secretion of these cytokines was assessed by ELISA (Figure 4.3). In response to LPS or zymosan, *Gata6*-deficient pMφs secreted significantly more TNF compared to pMφs from *Gata6*-WT mice ($p = 0.0002$ and $p < 0.001$ respectively) (Figure 4.3). Furthermore, TNF production by *Gata6*-deficient pMφ stimulated with LPS or zymosan was sustained, with significant

TNF production detected 24 hours post-stimulation. Conversely, in response to LPS or zymosan, TNF production by pMφs from *Gata6*-WT mice (stimulated with LPS or zymosan) was transient, peaking at 3 and 6 hours and undetectable 12 hours post stimulation. The significant increase in TNF secretion was consistent with the expression changes detected by qPCR. In contrast to the marked increase in *Il6* expression in *Gata6*-deficient pMφs stimulated with LPS for 3 hours detected by qPCR, the secretion of IL-6 detected by ELISA at 3 hours after stimulation with either LPS or zymosan was not significantly different between pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice ($p = 0.9999$ and $p = 0.9816$ respectively). The secretion of IL-6 *Gata6*-deficient pMφs stimulated with LPS for 24 hours was significantly higher than pMφs from *Gata6*-WT mice ($p = 0.0366$). At all other time points, the secretion of IL-6 detected in supernatants was not-significantly different between pMφs from *Gata6*-KO^{mye} compared to those from *Gata6*-WT mice stimulated with LPS or zymosan (Figure 4.3).

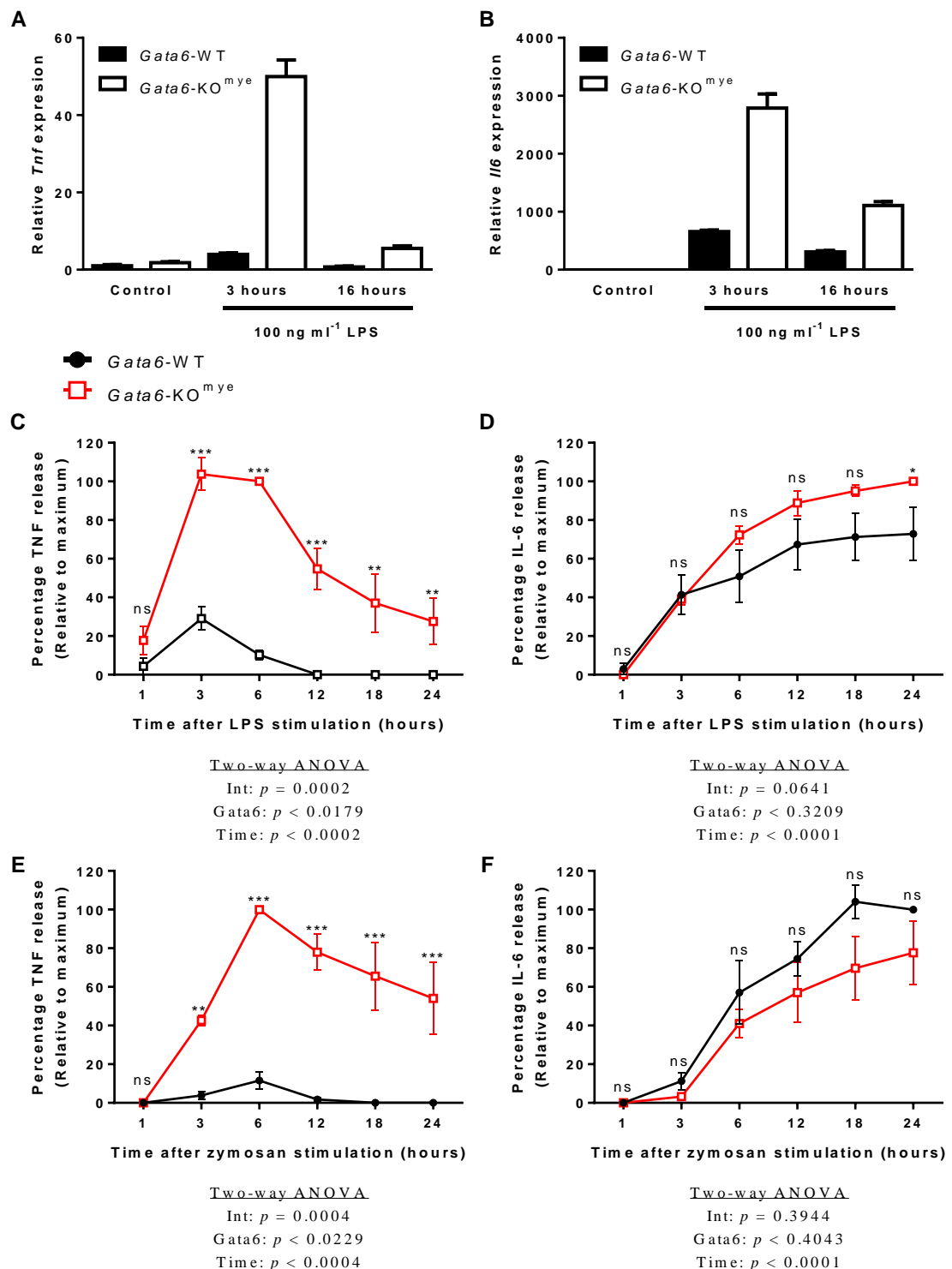


Figure 4.3 | *Gata6*-deficient pMφs have enhanced and sustained TNF production upon stimulation with LPS or zymosan.

(A, B) APECs from *Gata6*-WT and *Gata6*-KO^{mye} were unstimulated or stimulated with 100 ng ml⁻¹ LPS for 3 or 16 hours. The expression of *Tnf* and *Il6* were determined by qPCR relative to the endogenous control gene *Ywhaz*. Data is mean \pm 95% confidence interval from 3 technical replicates. (C-F) APECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were stimulated with 100 ng ml⁻¹ LPS (C-D) or a 1:1 ratio zymosan particles: cells (E-F) for the indicated time points. Supernatants were collected and analysed by ELISA for TNF (C, E) and IL-6 (D, F) release. Data are expressed as

mean \pm S.E.M as a percentage of release at 6 hours (TNF) and 24 hours (IL-6) from three independent experiments. Statistical significance (**C-F**) was determined by two-way ANOVA with Tukeys multiple comparison post-test (Int, interaction statistic; Gata6, Gata6 effects; Time, Treatment time effects) with Tukeys multiple comparison post-test (*p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant).

4.3.2 Aberrant TNF release is restricted to TLR4 stimulation

Gata6-deficient pMφs stimulated with LPS or zymosan produced significantly more TNF and for a sustained time-period compared to pMφs from *Gata6*-WT mice. To explore whether other TLR ligands could result in sustained TNF production, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice were either unstimulated or stimulated with different TLR ligands for 24 hours after which the release of TNF and IL-6 were determined by ELISA (Figure 4.4).

In contrast to LPS stimulation alone which resulted in significant TNF release from *Gata6*-deficient pMΦs after 24 hours ($p < 0.0001$), none of the other TLR ligands tested resulted in significant differences in TNF release from *Gata6*-WT and *Gata6*-KO^{mye} mice (Figure 4.4). The production of IL-6 was not significantly different between pMφs from *Gata6*-WT and *Gata6*-KO^{mye} stimulated with different TLR ligands.

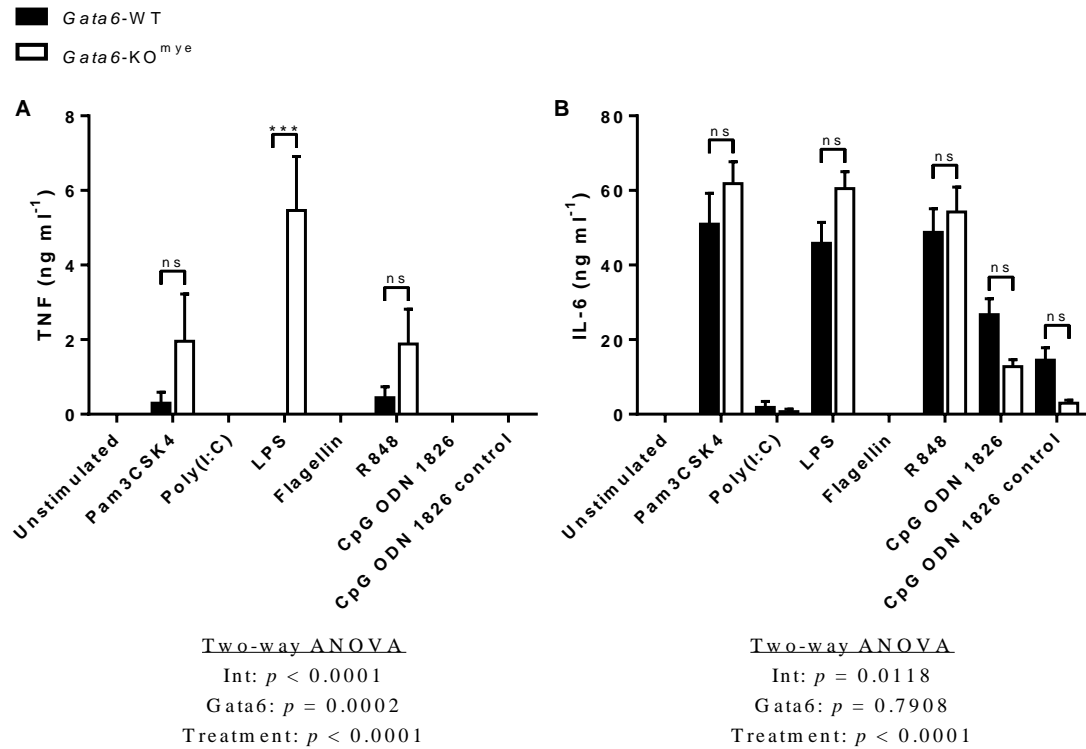


Figure 4.4 | Aberrant TNF release from *Gata6*-deficient pMφs is restricted to TLR4 stimulation.

(A, B) APECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were unstimulated or stimulated with Pam3CSK4 (500 ng ml⁻¹), Poly I:C (1 μg ml⁻¹), LPS (100 ng ml⁻¹), Flagellin (100 ng ml⁻¹), R848 (1 μg ml⁻¹), CpG ODN 1826 (5 μM) or CpG ODN 1826 control (5 μM) for 24 hours. Culture supernatants were collected 24 hours after stimulation and (A) TNF and (B) IL-6 release measured by ELISA. Data are shown as mean ± S.E.M from five independent experiments. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Gata6, *Gata6* effects; Treatment, Treatment effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3.3 Intracellular cytokine staining reveals that IL-6 is significantly decreased in *Gata6*-deficient pMφs stimulated with LPS

The composition of cells within the peritoneal cavity of *Gata6*-KO^{mye} mice is markedly different to that of *Gata6*-WT mice. The number of resident pMφs is dramatically reduced from approximately 50% in *Gata6*-WT mice to 20% in *Gata6*-KO^{mye} mice (Rosas et al., 2014, Okabe and Medzhitov, 2014). The loss in resident pMφs in *Gata6*-KO^{mye} is accompanied by an expansion/increase in the number of eosinophils and MHCII^{high}CD226⁺ DC-like cells.

To determine the contribution of pMφs to inflammatory cytokine production, and exclude the possibility of other cell subsets contributing to the differences in cytokine production detected by ELISA, the production of TNF and IL-6 in unstimulated and LPS-stimulated pMφs from *Gata6*-WT and *Gata6*-KO^{mye} was determined by intracellular flow cytometry (Figure 4.5).

In accordance with the gene expression and ELISA, TNF production was significantly increased pMφs from *Gata6*-KO^{mye} compared to *Gata6*-WT mice stimulated with LPS for 3 hours ($p < 0.0001$) (Figure 4.5). Surprisingly, the production of IL-6 in LPS-stimulated pMφs from *Gata6*-KO^{mye} was significantly lower compared to *Gata6*-WT mice ($p < 0.0001$), which is in stark contrast to the expression and release of IL-6 detected by ELISA (Figure 4.5).

Interestingly, the production of TNF by LPS-stimulated pMφs from *Gata6*-WT and F4/80^{high} pMφs from *Gata6*-KO^{mye} mice was comparable ($p = 0.9977$), whereas the TNF production was markedly higher in F4/80^{low} pMφs from *Gata6*-KO^{mye} ($p < 0.0001$). Furthermore, the production of IL-6 in F4/80^{high} pMφs was significantly higher than in F4/80^{low} pMφs ($p < 0.0001$), however, this was lower than pMφs from *Gata6*-WT mice ($p = 0.0027$) (Figure 4.5).

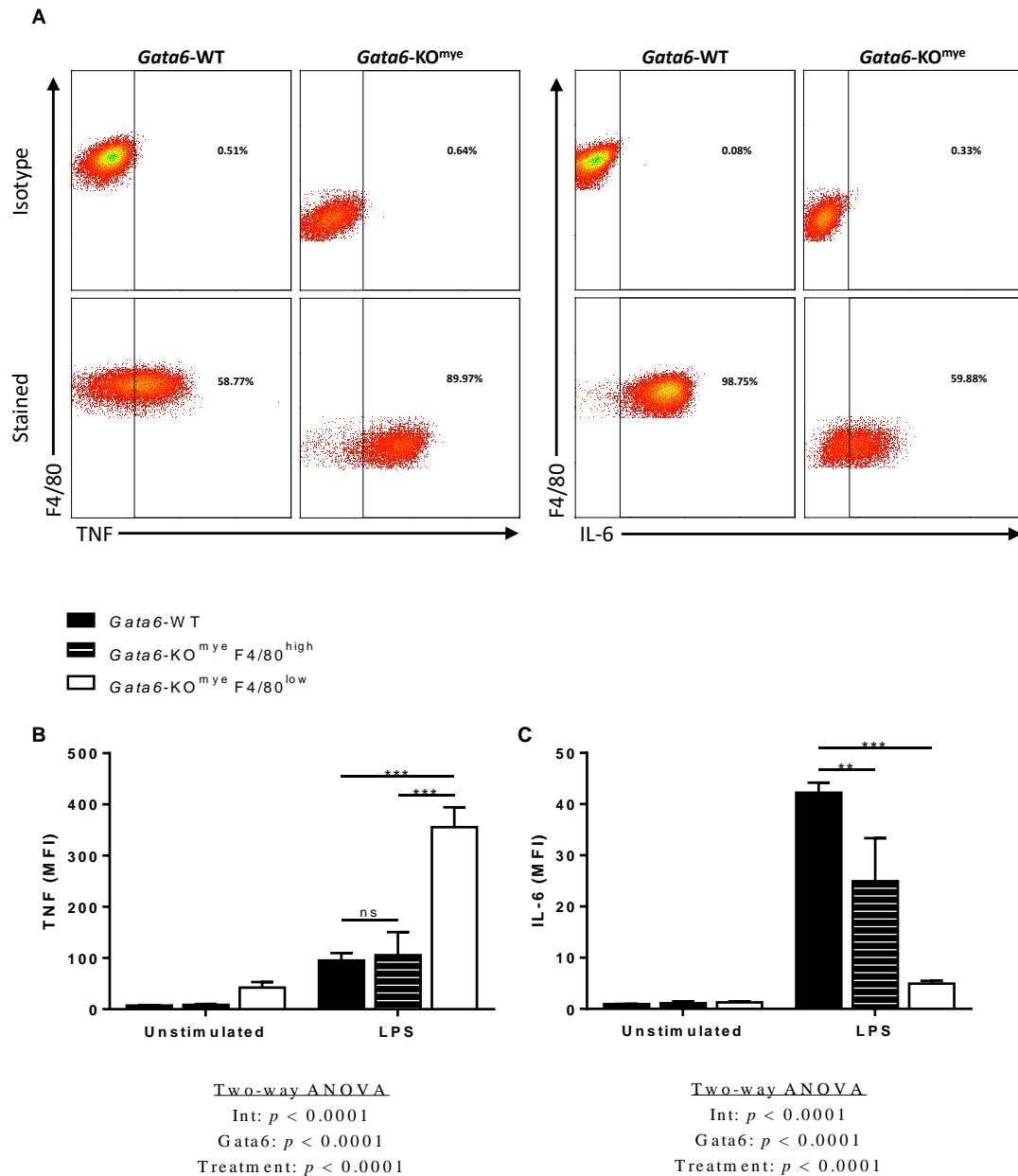


Figure 4.5 | *Gata6*-deficient pMφs have enhanced TNF and reduced IL-6 in response to LPS.

(A-C) Total PECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were unstimulated or stimulated with 100 ng ml⁻¹ LPS for 3 hours in the presence of GolgiBlock. (A) Representative density/bivariate plot of IL-6 and TNF production by pMφs (gated by F4/80⁺Tim4⁺) was measured by intracellular cytokine staining. (B, C) Bar chart shows mean MFI (minus isotype control) of TNF and IL-6 production in Tim4⁺F4/80^{high} pMφs from *Gata6*-WT mice and Tim4⁺F4/80^{high} and Tim4⁺F4/80^{low} pMφ populations within the same *Gata6*-KO^{mye} mice. Data shows mean ± S.D from 2-4 individual mice per group from two independent experiments. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Gata6, Gata6 effects; Treatment, Treatment effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3.4 Co-cultured *Gata6*-WT pMφs prevent aberrant TNF release from *Gata6*-deficient pMφs

The release of IL-1 β from LPS-stimulated *Gata6*-deficient pMφs was significantly reduced when co-cultured with pMφs from *Gata6*-WT mice. To determine whether the aberrant production of TNF could be restored by pMφs from *Gata6*-WT mice, the release of TNF from monocultured cells and co-cultured was determined in LPS-stimulated pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice (Figure 4.6).

The secretion of TNF from LPS-stimulated *Gata6*-KO^{mye} monocultures seeded 1×10^5 cells was not significantly different to those containing 0.5×10^5 *Gata6*-deficient pMφs ($p = 0.7506$). Co-cultured pMφs released significantly less TNF (not detectable) than monocultures containing 0.5×10^5 *Gata6*-deficient pMφs ($p = 0.0003$). Conversely, the release of IL-1 β from co-cultured pMφs was not significantly different to monocultures containing 0.5×10^5 pMφs from *Gata6*-WT mice ($p > 0.9999$) (Figure 3.14).

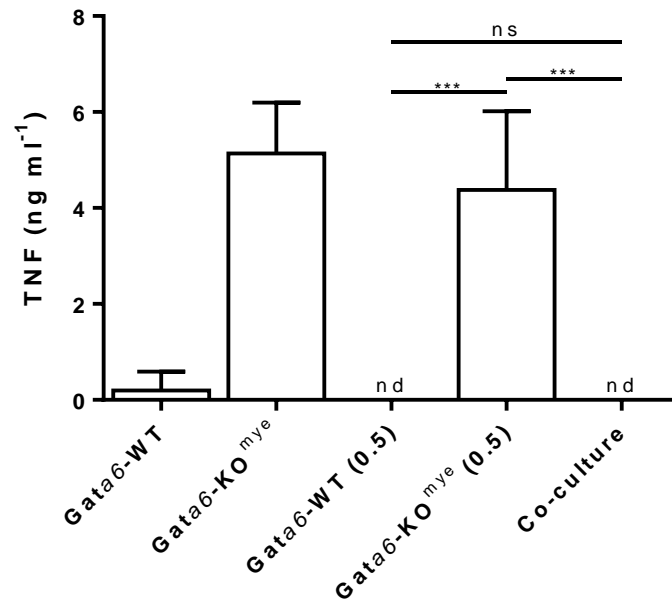


Figure 4.6 | Aberrant LPS-induced TNF release from *Gata6*-deficient pMφs is attenuated when co-cultured with pMφs from *Gata6*-WT mice.

APECs from *Gata6*-WT and *Gata6*-KO^{mye} were cultured separately (1×10^5 and 0.5×10^5) or co-cultured at a 1:1 ratio (1×10^5 total cells) after which they were stimulated with 100 ng ml^{-1} LPS for 24 hours. After stimulation, TNF release was determined in cell-free culture supernatants by ELISA. Data shows mean \pm S.D from four independent experiments. nd = not detectable. Statistical significance was determined by one-way ANOVA with Tukeys multiple comparison post-test between co-cultured cells and monocultures containing 0.5×10^5 cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

4.3.5 Prostacyclin synthase expression is selectively expressed in pMφs and is dependent on Gata6

Having established that the release of TNF from LPS-stimulated *Gata6*-deficient pMφs was attenuated when co-cultured with pMφs from *Gata6*-WT mice, the mechanisms through which Gata6 may modulate inflammatory responses were investigated. Analysis of microarray data showed that one of the most highly down-regulated genes in *Gata6*-deficient pMφs was prostacyclin synthase (*Ptgis*), the key enzyme required for PGI₂ production ($p = 0.003502$) (Figure 4.7). Furthermore, western blotting showed that *Ptgis* expression was greatly reduced in *Gata6*-deficient pMφs (Figure 4.7). The expression of *Ptgis* was analysed in different immune cell populations, which revealed that it was predominantly restricted to resident pMφs (Figure 4.7).

To determine whether the Gata6 regulates the expression of other terminal synthases in pMφs (which would influence the balance of prostanoids produced) microarray expression data from *Gata6*-WT and *Gata6*-KO^{mye} mice was analysed (Figure 4.7). Interestingly, whilst the expression of other terminal synthases was not significantly different, thromboxane synthase (*Tbxas1*) was significantly increased in *Gata6*-deficient pMφs ($p = 0.005213$) (Figure 4.7). Thromboxane synthase is the critical enzyme mediating the synthesis of thromboxane (TXA₂), which exerts biologically effects opposing the actions of PGI₂ (Caughey et al., 1997). PGI₂ has been reported to signal via two receptors; autocrine and paracrine signalling through the prostacyclin receptor (IP); and intracrine signalling mediated through peroxisome proliferator activated receptors (PPARs) on nuclear membranes (Lim and Dey, 2002, Li et al., 2012, Hertz et al., 1996, Forman et al., 1997). Microarray analysis of pMφs from *Gata6*-WT and *Gata6*-KO^{mye} showed that the expression of PPARβ/δ, PPARγ, and IP receptor expression were unaffected by *Gata6*-deficiency (Figure 4.7).

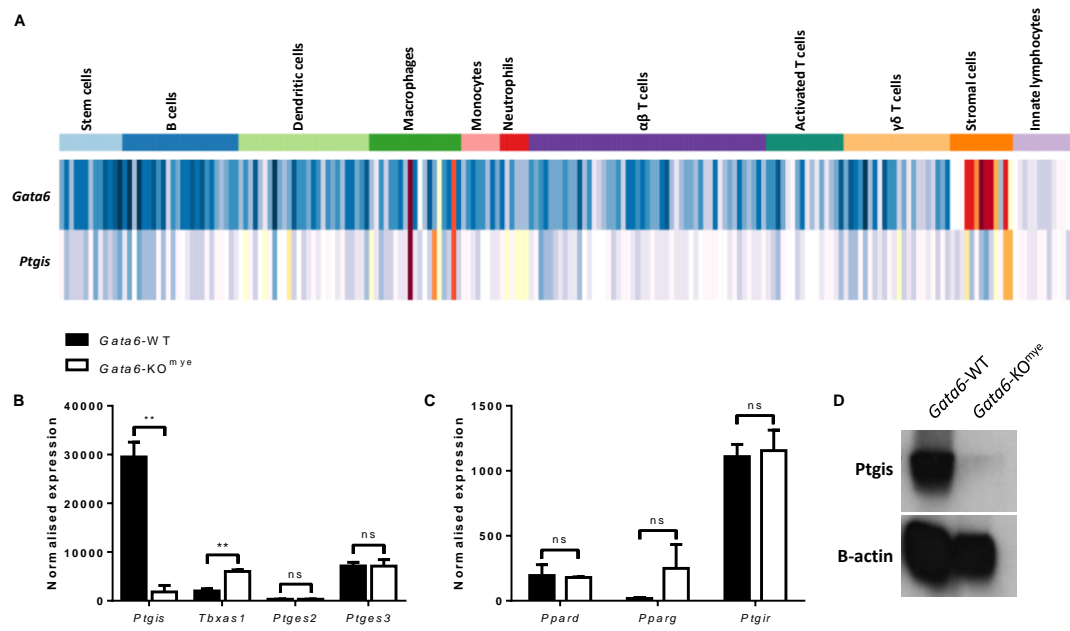


Figure 4.7 | *Ptgis* is highly expressed in resident peritoneal macrophages and dependent on *Gata6*.

(A) Heatmap showing the expression of *Gata6* and *Ptgis* across different immune cell populations. Data was generated and provided by the ImmGen consortium. (B, C) Microarray expression of terminal prostanoïd synthases (B) *Ptgis*, thromboxane synthase (*Tbxas1*), prostaglandin E synthase 2 and 3 (*Ptges1* and *Ptges2*) and PGI₂ receptors (C) PPAR β/δ , PRR γ and *Ptgir* in pMφs from *Gata6*-WT and *Gata6*-KO^{mye}. Data is expressed as mean \pm S.D from three biological replicates. (D) Immunoblot showing *Ptgis* and β -actin in pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice. Image representative of two independent experiments. Statistical significance (B, C) was determined using an empirical Bayesian statistic corrected for false discovery rate by the Benjamini-Hochberg procedure. Not significant (ns) $p \geq 0.5$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3.6 Prostacyclin analogues modulate TNF and IL-6 production in pMφs.

To examine how prostanoids effected the production of pro-inflammatory cytokines, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice were either unstimulated, stimulated with LPS alone, or stimulated with LPS in the presence of beraprost or indomethacin and analysed by flow cytometry for TNF and IL-6 (Figure 4.8).

In accordance with my previous findings, *Gata6*-deficient pMφs produced significantly more TNF and significantly less intracellular IL-6 compared to pMφs from *Gata6*-WT mice (Figure 4.8). Upon treatment with the PGI₂ analogue beraprost, the production of TNF was significantly reduced in pMφs from *Gata6*-WT mice and F4/80^{low} and F4/80^{high} pMφs from *Gata6*-KO^{mye} mice (Figure 4.8). The production of TNF by pMφs from *Gata6*-WT and F4/80^{high} pMφs from *Gata6*-KO^{mye} mice was greatly increased when cells were stimulated with LPS and indomethacin compared to LPS alone ($p = 0.0023$ and $p = 0.0342$ respectively). Conversely, the increase in TNF production from F4/80^{low} pMφs treated with LPS and indomethacin was comparable to LPS treatment alone ($p > 0.9999$) (Figure 4.8).

The production of IL-6 was significantly increased in *Gata6*-deficient pMφs treated with LPS and beraprost compared to LPS treatment alone ($p = 0.0048$). Conversely, pMφs from *Gata6*-WT mice treated with LPS and beraprost had no significant effect on the production of IL-6 compared to treatment with LPS alone ($p = 0.8177$). The production of IL-6 by pMφs from *Gata6*-WT mice was significantly reduced when treated with LPS and indomethacin compared to LPS treatment alone ($p = 0.0005$). Treatment of *Gata6*-deficient pMφs with LPS and indomethacin had no discernible effect on IL-6 production compared to LPS treatment alone ($p > 0.9999$). The production of IL-6 in F4/80^{high} pMφs from *Gata6*-KO^{mye} was comparable to treatment with LPS alone ($p = 0.2407$).

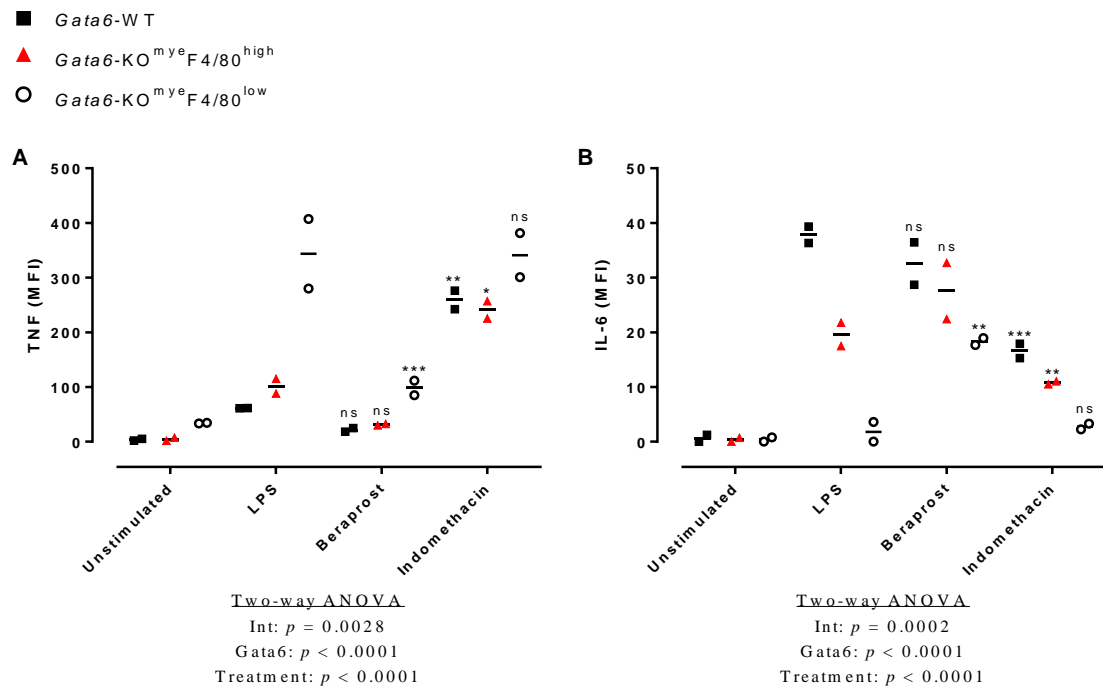


Figure 4.8 | Prostacyclin analogues regulate TNF and IL-6 expression.

(A, B) Total PECs from *Gata6*-WT and *Gata6*-KO^{mye} mice were unstimulated or stimulated with 100 ng ml⁻¹ LPS for 3 hours in the presence of DMSO (vehicle), 10 μM Beraprost or 10 μM Indomethacin. Scatter plots shows mean MFI (minus isotype control) of TNF and IL-6 production in Tim4⁺F480⁺ pMφs from *Gata6*-WT and Tim4⁺F4/80^{high} and Tim4⁺F4/80^{low} populations within the same *Gata6*-KO^{mye} mice. Data shows MFI from two independent experiments. Statistical significance was determined by two-way ANOVA between LPS stimulation alone and with the indicated compounds (Int, interaction statistic; Gata6, Gata6 effects; Treatment, Treatment effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

4.3.7 Prostacyclin synthase (*Ptgis*) transgene expression

The restoration of normal inflammatory responses in LPS-stimulated *Gata6*-deficient pMφs through treatment with beraprost supported the hypothesis that the loss of *Ptgis* expression in pMφs led to dysregulated cytokine production upon stimulation with LPS.

To determine how *Ptgis* expression effects the inflammatory response, the next aim was to reconstitute *Ptgis* expression in *Gata6*-deficient pMφs through *Ptgis*-expressing lentiviral constructs. To address this, *Ptgis* (accession NM_008968) was cloned from the cDNA of WT pMφs and inserted into a lentiviral vector containing the reporter rCD2 and T2A peptide (*T2A-rCD2* (Figure 4.9)). To assess the functionality of the *Ptgis* transgene, BMDMs were non-transduced (WT) or transduced with lentivirus expressing *Ptgis* or the *T2A-rCD2* (empty vector control) constructs. The expression of *Ptgis* was determined 72 hours post transduction by qPCR and immunoblotting (Figure 4.9).

The expression of *Ptgis* in BMDMs was undetectable by either qPCR or immunoblotting, supporting microarray data from the ImmGen consortium showing that *Ptgis* expression is largely restricted to pMφs (Figure 4.9). Similarly, BMDMs transduced with empty vector control (*T2A-rCD2*) had limited detectable *Ptgis* expression, whereas transduction with the *Ptgis* transgene greatly increased the expression of *Ptgis* (Figure 4.9).

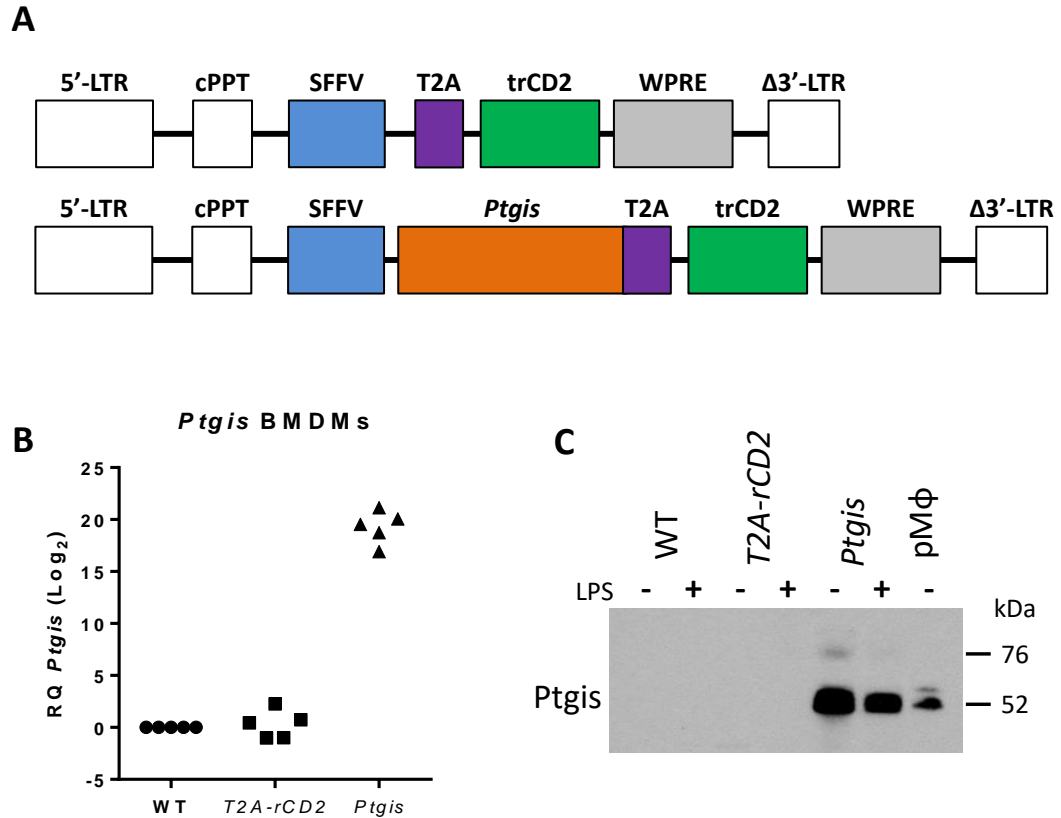


Figure 4.9 | *Ptgis* transgene expression in BMDMs.

(A) Schematic of *Ptgis*-expressing and empty vector control (*T2A-rCD2*) lentiviral constructs. (B, C) BMDMs were either non-transduced (WT) or transduced with *Ptgis* or *T2A-rCD2* control expressing lentiviruses. (B) *Ptgis* expression was determined by qPCR relative to the endogenous control gene *Ywhaz*. Scatter dot plot shows Log_2 expression from five biological replicates (individual points) from three independent experiments. (C) Immunoblot of showing *Ptgis* expression in BMDMs unstimulated or stimulated with LPS for 24 hours. Peritoneal macrophages (pMφs) were used as a positive control for *Ptgis* expression. Image representative of two independent experiments.

4.3.8 BMDM expressing *Ptgis* transgene constructs produce PGI₂

Having established that the *Ptgis* transgene was expressed by BMDMs, the functionality of the protein was assessed. Therefore, BMDMs either non-transduced (WT) or transduced with *T2A-rCD2*- or *Ptgis*-expressing lentiviruses were either unstimulated or stimulated with LPS for 24 hours. The supernatants were analysed by mass spectrometry for PGD₂, PGE₂, TXB₂ and 6-keto-PGF1 α release (Figure 4.10).

The production of prostanoids in unstimulated BMDMs was very low/undetectable and comparable between WT BMDMs and those transduced with either *T2A-rCD2*- or *Ptgis*-expressing lentiviruses (Figure 4.10). However, upon stimulation with LPS, a marked increase in 6-Keto-PGF1 α (a stable metabolite of PGI₂) was detected in supernatants of BMDMs transduced with *Ptgis*-expressing compared to WT and *T2A-rCD2* transduced BMDMs (Figure 4.10). Furthermore, the production of PGD₂, PGE₂ and TXB₂ (a stable metabolite of TXA₂) were reduced in LPS-stimulated BMDMs expressing *Ptgis* compared to WT and *T2A-rCD2* BMDMs (Figure 4.10).

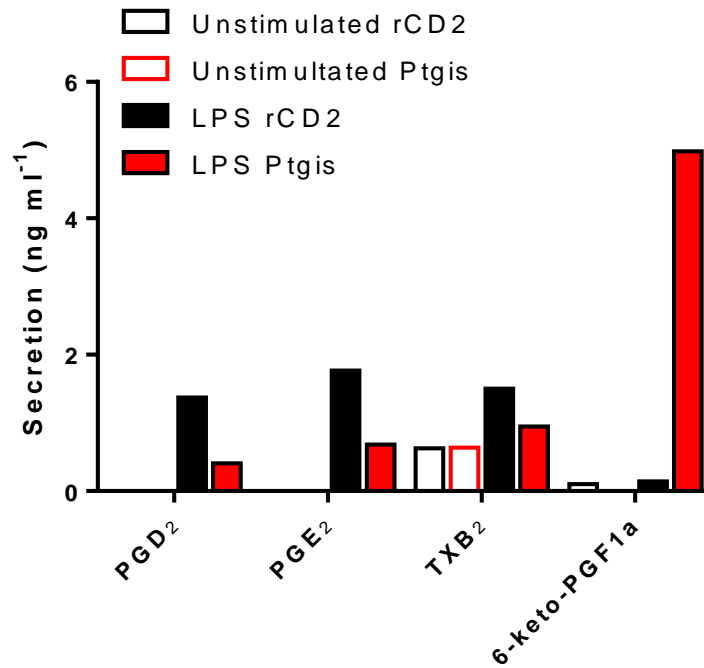


Figure 4.10 | Prostacyclin release is greatly enhanced in LPS-stimulated BMDMs transduced with *Ptgis* transgene.

BMDMs either non-transduced (WT) or transduced with either *T2A-rCD2*- or *Ptgis*-expressing lentiviral constructs and either unstimulated or stimulated with 100 ng ml⁻¹ for 24 hours. The supernatants were analysed by mass spectrometry for PGD₂, PGE₂, TXB₂ and 6-keto-PGF_{1α} release. Data are representative of two independent experiments.

4.3.9 Pro-inflammatory cytokine production by BMDMs is greatly reduced by PGI₂ analogue but not *Ptgis* transgene

To examine whether the production of pro-inflammatory cytokines is modulated by the expression of *Ptgis* transgene, BMDMs that were non-transduced or transduced with *T2A-rCD2* or *Ptgis*-expressing lentiviruses were unstimulated or stimulated with LPS and the production of TNF and IL-6 determined by intracellular flow cytometry (Figure 4.11).

The production of IL-6 was barely detectable in both unstimulated and LPS-stimulated BMDMs that were non-transduced or transduced with *T2A-rCD2* or *Ptgis* (Figure 4.11). Conversely, the production of TNF was significantly increased in BMDMs stimulated with LPS for 3 hours compared to unstimulated controls (Figure 4.11). The expression of the *Ptgis* transgene did not significantly affect the production of TNF compared to non-transduced or *T2A-rCD2* transduced BMDMs (Figure 4.11).

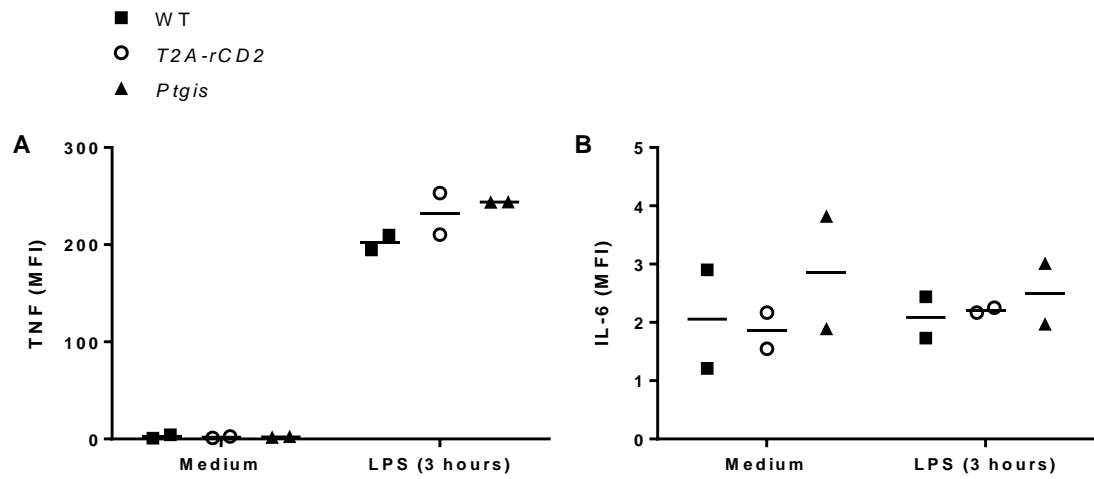


Figure 4.11 | Pro-inflammatory cytokine production is unaffected by *Ptgis* transgene expression.

BMDMs were non-transduced (WT) or transduced with lentivirus expressing *Ptgis* or *T2A-rCD2* (empty vector control). BMDMs were subsequently untreated (medium) or treated with 100 ng ml⁻¹ LPS for 3 hours (in the presence of GolgiBlock), after which the production of TNF was assessed by flow cytometry. Data shows MFI (minus isotype control) from two biological replicates.

The production of TNF in BMDMs stimulated with LPS for 3 hours was unaffected by Ptgis transgene expression. To examine whether Ptgis transgene can modulate pro-inflammatory cytokine production over extended time periods, BMDMs that were non-transduced or transduced with either *T2A-rCD2* or Ptgis were unstimulated or stimulated with LPS for 6 hours and the production of IL-6 and TNF determined by intracellular flow cytometry (Figure 4.12).

The production of TNF by BMDMs stimulated with LPS for 6 hours was not significantly different between BMDMs that were non-transduced or transduced with either *T2A-rCD2* or Ptgis ($p = 0.2270$ and $p = 0.1384$ respectively) (Figure 4.12). Furthermore, IL-6 production from BMDMs after 6 hours stimulation with LPS was very low.

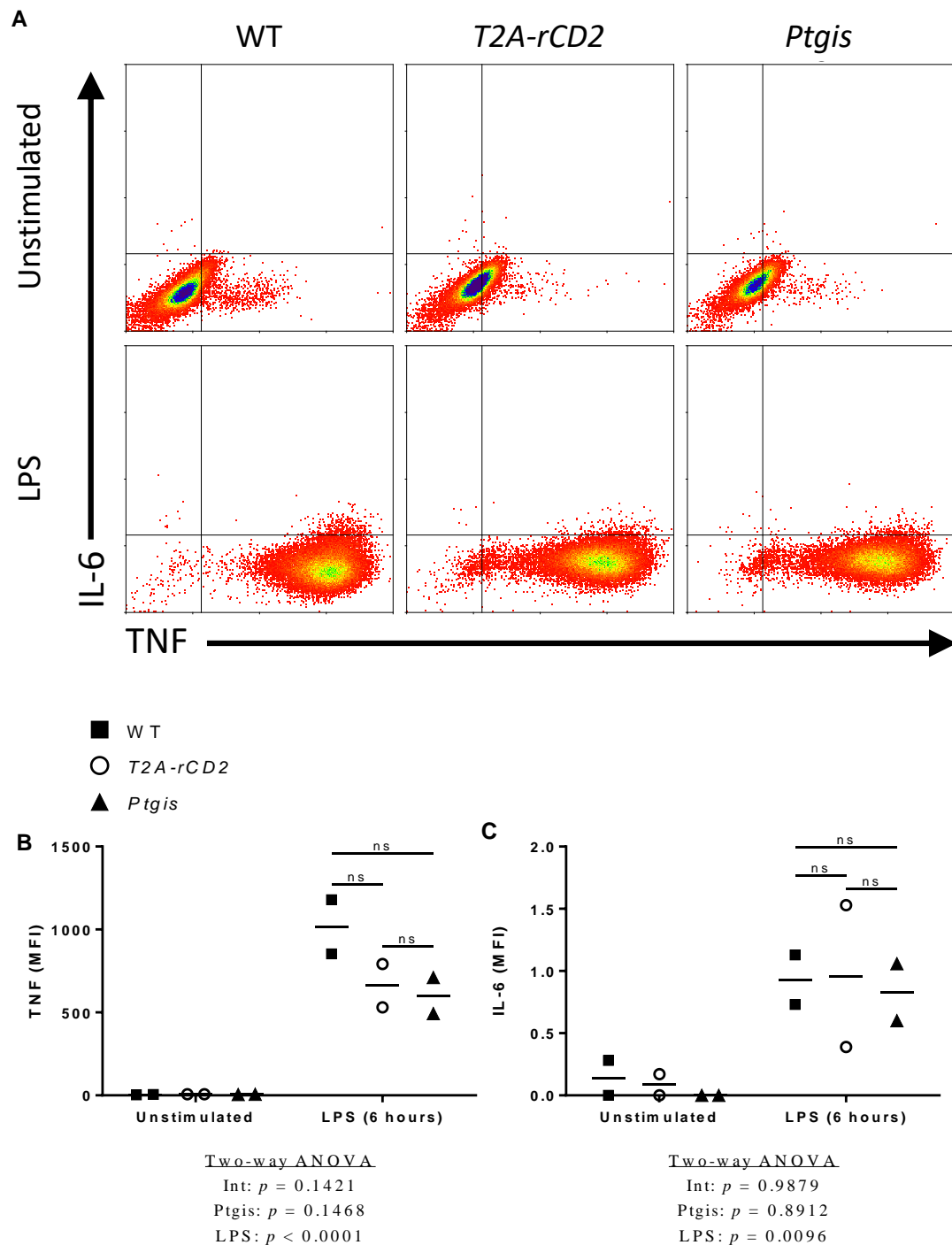


Figure 4.12 | *Ptgis* transgene expression doesn't attenuate pro-inflammatory cytokine production.

A) Representative bivariate plots showing intracellular cytokine of TNF and IL-6 in BMDMs that were non-transduced (WT) or transduced with lentivirus expressing *T2A-rCD2* (empty vector) or *Ptgis* that were either unstimulated or stimulated with 100 ng ml^{-1} LPS for 6 hours. (B, C) Scatter plots show mean MFI (minus isotype control) of TNF and IL-6 production from two independent experiments. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Ptgis, Ptgis effects; LPS, LPS effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

To determine whether the production of pro-inflammatory mediators can be modulated by prostanoids, BMDMs were either unstimulated or stimulated with LPS alone or LPS in the presence of indomethacin or beraprost for 3 hours and the production of IL-6 and TNF determined by intracellular flow cytometry (Figure 4.13).

The production of TNF in BMDMs was significantly lower in response to treatment with LPS and beraprost compared to treatment with LPS alone (Figure 4.13 B). Interestingly, the production of TNF by BMDMs treated with LPS and indomethacin was not significantly different to LPS treatment alone (Figure 4.13 B). The production of IL-6 treated with LPS alone and in the presence of either indomethacin or beraprost was not significantly different from unstimulated BMDMs (Figure 4.13 C).

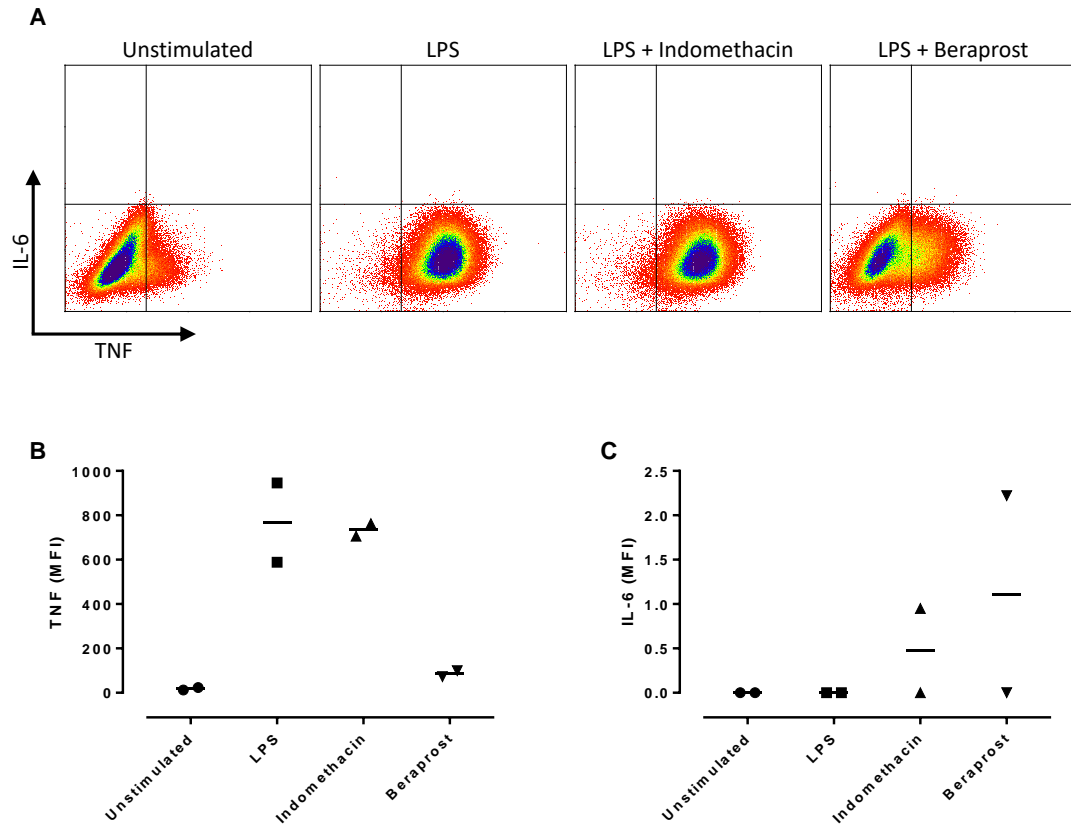


Figure 4.13 | PGI₂ analogues reduce TNF production in BMDMs.

(A-C) BMDMs were unstimulated or stimulated with 100 ng ml⁻¹ LPS with or without 10 μM indomethacin or 10 μM beraprost for 3 hours in the presence of GolgiBlock and the production of TNF and IL-6 measured by intracellular cytokine staining. (A) Representative bivariate plot of TNF and IL-6 production. (B, C) Scatter plots show TNF and IL-6 MFI (minus isotype control) from two independent experiments.

4.3.10 *In vivo* transduction of resident peritoneal macrophages with PTGIS-expressing lentivirus

To elucidate how PGI₂ regulates the production of pro-inflammatory cytokines in pMφs, *Gata6*-WT and *Gata6*-KO^{mye} mice were injected intraperitoneally with lentivirus expressing *T2A-rCD2* or *Ptgis*. The cells were unstimulated or stimulated with LPS *ex vivo* and the production of TNF and IL-6 determined by intracellular flow cytometry (Figure 4.14).

- T2A-*rCD2* (*rCD2*⁻)
- T2A-*rCD2* (*rCD2*⁺)
- △ *Ptgis* (*rCD2*⁻)
- ▼ *Ptgis* (*rCD2*⁺)

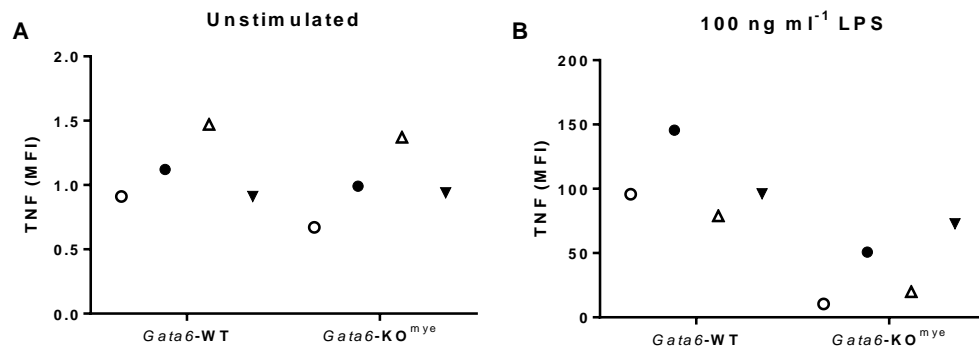


Figure 4.14 | Reconstitution of *Ptgis* expression in *Gata6*-KO^{mye} fails to attenuate inflammatory cytokine production.

Gata6-WT *Gata6*-KO^{mye} mice were transduced with *T2A-rCD2* or *Ptgis* by intraperitoneal injection with lentivirus. After 7 days, total peritoneal cells were harvested and either (A) unstimulated or (B) stimulated with 100 ng ml⁻¹ LPS for 3 hours. The production of TNF was determined by intracellular flow cytometry in CD11b⁺F4/80⁺ pMφs that were either transduced (*rCD2*⁺) or non-transduced (*rCD2*⁻). Data shows MFI TNF expression from one experiment.

4.3.11 Does the knockdown of *Ptgis* expression result in dysregulated pro-inflammatory cytokine production?

To corroborate the *Ptgis* reconstitution experiments, resident pMφs were transduced *in vivo* by intraperitoneal injection of lentiviral shRNA constructs targeting *Ptgis* (sh*Ptgis* #1 and #2) and a non-silencing control (shNSC) construct. The expression of *Ptgis* was determined by qPCR in FACS-sorted pMφs (F4/80⁺CD11b⁺MHCII⁻) that were transduced (rCD2⁺) and non-transduced (rCD2⁻) with lentiviral constructs (Figure 4.15).

The expression of *Ptgis* was not significantly different between non-transduced and shNSC transduced pMφs. However, the expression of *Ptgis* was down-regulated by approximately 78% and 85% by sh*Ptgis* #1 and #2 respectively compared to the non-transduced pMφs isolated from the same mouse (Figure 4.15).

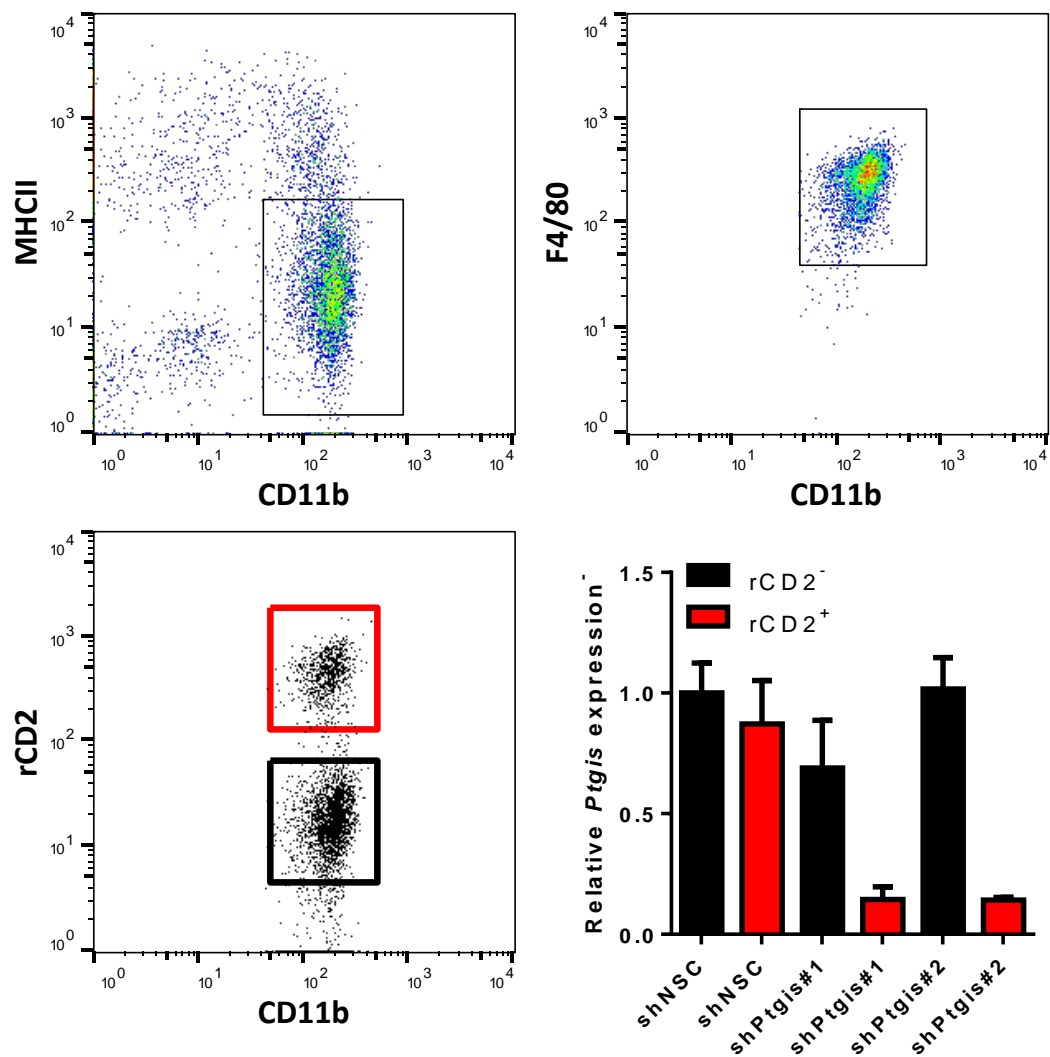


Figure 4.15 | *In vivo* lentiviral knockdown of *Ptgis* in pMφs.

C57BL/6 mice were injected intraperitoneally with two lentiviral shRNA constructs targeting *Ptgis* (shPtgis #1 and #2) and a non-silencing control (shNSC). After 7 days, peritoneal cells were harvested and sorted by FACS. pMφs (CD11b⁺ F4/80⁺ MHCII⁻) cells were sorted into two populations, non-transduced (rCD2⁻) and transduced cells (rCD2⁺). The expression of *Ptgis* in each population was determined by qPCR relative to the endogenous control *Ywhaz*. Error bars represent mean \pm S.D from 3 technical replicates. ($n = 1$).

4.3.12 *Ptgis* knockdown doesn't affect pro-inflammatory cytokine expression

To investigate how PGI₂ controls the production of pro-inflammatory cytokine production, pMφs were transduced *in vivo* with lentivirus expressing shNSC or sh*Ptgis* constructs. Total peritoneal cells were either unstimulated or stimulated with 100 ng ml⁻¹ LPS *ex vivo* and the production of IL-6 and TNF determined by intracellular cytokine staining (Figure 4.16).

The production of TNF by pMφs stimulated with LPS for three hours was not significantly different between pMφs transduced (rCD2⁺) with shNSC- or sh*Ptgis*-expressing lentivirus ($p = 0.8468$). The production of TNF between transduced and non-transduced pMφs stimulated with LPS for three hours was not significantly different in mice injected with either shNSC- or sh*Ptgis*-expressing lentivirus ($p = 0.8232$ and $p = 0.2192$ respectively). Similarly, the production of IL-6 was comparable between pMφs transduced with shNSC- and sh*Ptgis*-expressing lentivirus stimulated with LPS for three hours ($p = 0.9267$) (Figure 4.15). Likewise, IL-6 production from transduced and non-transduced pMφs stimulated with LPS for three hours was not significantly different in mice injected with either shNSC- or sh*Ptgis*-expressing lentivirus ($p = 0.8102$ and $p = 0.9932$ respectively) (Figure 4.15).

The production of both IL-6 and TNF were drastically reduced after 6 hours stimulation with LPS (GolgiBlock added at 3 hours) compared to stimulation with LPS for 3 hours. The production of TNF by pMφs stimulated with LPS for six hours was not significantly different between pMφs transduced (rCD2⁺) with shNSC- or sh*Ptgis*-expressing lentivirus ($p > 0.9999$). Similarly, TNF production between transduced and non-transduced pMφs stimulated with LPS for six hours was comparable in mice injected with either shNSC- or sh*Ptgis*-expressing lentivirus ($p > 0.9999$ and $p > 0.9999$ respectively). Conversely, pMφs transduced with sh*Ptgis* and stimulated for 6 hours with LPS had significantly less IL-6 production than pMφs transduced with shNSC ($p = 0.0084$). IL-6 production from transduced and non-transduced pMφs stimulated with LPS for six hours was not significantly different in mice injected with either shNSC- or sh*Ptgis*-expressing lentivirus ($p = 0.2349$ and $p = 0.9805$ respectively).

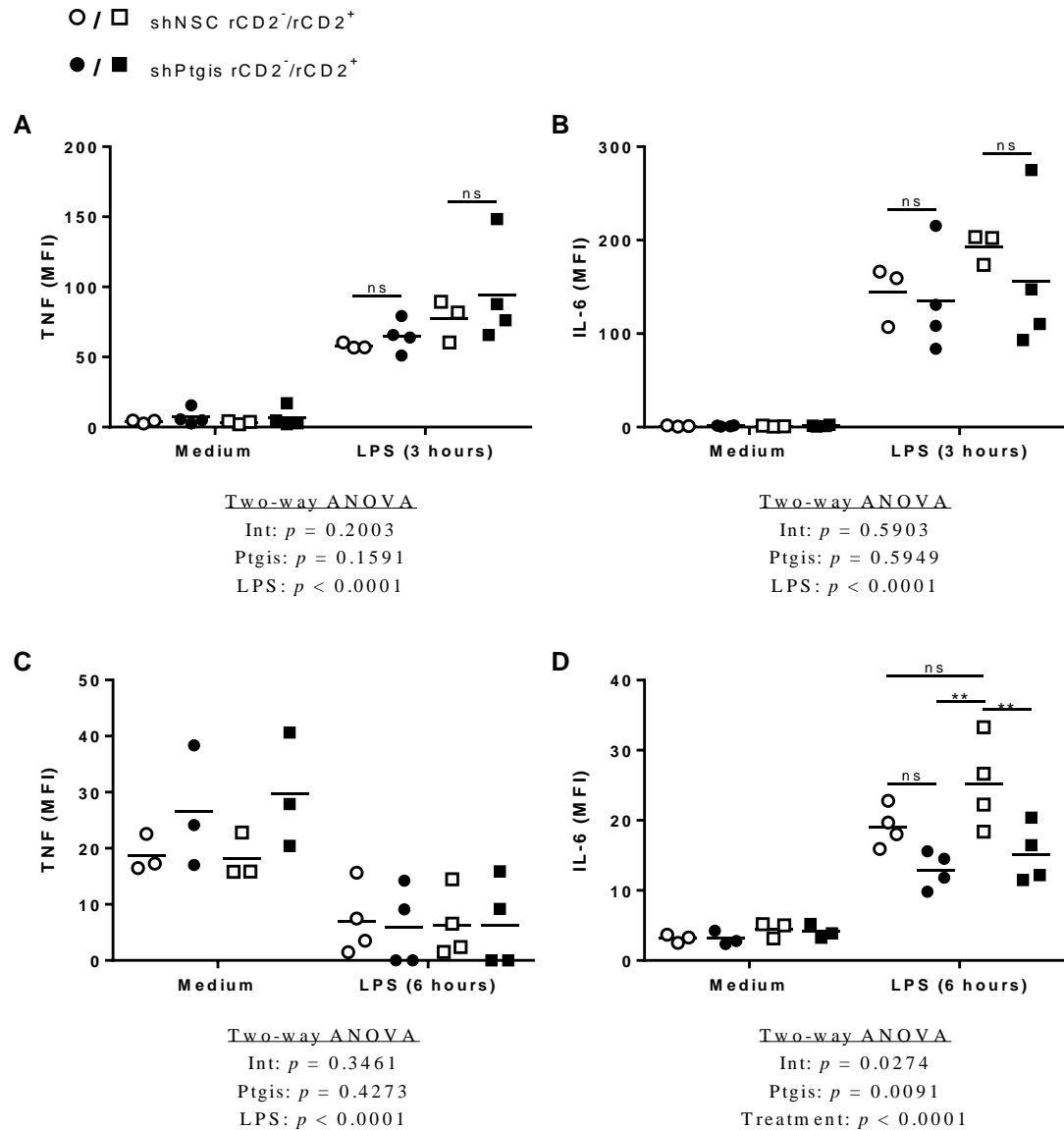


Figure 4.16 | *Ptgis* knockdown doesn't modulate pro-inflammatory cytokine production.

(A – D) C57BL/6 mice were injected intraperitoneally with lentiviral shRNA constructs targeting *Ptgis* (shPtgis) and a non-silencing control (shNSC). After 7 days, total peritoneal cells were harvested and either unstimulated or stimulated with 100 ng ml⁻¹ LPS for (A, B) 3 or (C, D) 6 hours. The production of TNF and IL-6 was determined by intracellular flow cytometry in non-transduced (rCD2⁻) and transduced (rCD2⁺) pMφs (F4/80⁺CD11b⁺) from the same mouse. Scatter plots show MFI (minus isotype control) of TNF (A, C) and IL-6 (B, D). (n = 3-4 mice per group). Data representative of one independent experiment. Statistical significance was determined by two-way ANOVA (Int, interaction statistic; Ptgis, Ptgis effects; LPS, LPS effects) with Tukeys multiple comparison post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.4 Discussion

4.4.1 *Gata6*-deficient pMφs have perturbed production of pro-inflammatory cytokines

Preliminary experiments by Dr Marcela Rosas suggested that *Gata6*-deficient pMφs may have perturbed inflammatory responses. To elucidate the role *Gata6* has on the inflammatory response, pMφs were stimulated with an array of microbial ligands and the expression of pro-inflammatory genes determined by qPCR and the production determined by ELISA and intracellular flow cytometry. Strikingly, the expression and release of TNF was significantly increased in *Gata6*-deficient pMφs (Figure 4.3). TNF is a potent cytokine, whose dysregulation has been implicated in numerous inflammatory conditions. Interestingly, whilst the expression and release of TNF was transient in pMφs from *Gata6*-WT mice, *Gata6*-deficient pMφs exhibited sustained TNF expression and secretion, implicating a role for *Gata6*, directly or indirectly, in promoting the resolution of inflammation. Stimulation with various TLR ligands revealed that the aberrant pro-inflammatory cytokine production was predominantly TLR4-dependent, although zymosan, also induced robust defects similar to LPS stimulation. The selectivity of TLR ligands may be due to restricted TLR expression, which was apparent flagellin in particular, which did not produce IL-6 at a range of concentrations (Supplementary Figure 2 and Supplementary Figure 3). Alternatively, other ligands may induce significant pro-inflammatory cytokine production at earlier time points, for which this experiment only analysed supernatants after 24 hours of stimulation.

Conversely, whilst qPCR and ELISA data suggested that IL-6 production was significantly enhanced in LPS-stimulated *Gata6*-deficient pMφs, intracellular flow cytometry showed that they have reduced production compared to pMφs from *Gata6*-WT mice (Figure 4.5). These discrepancies may arise from distinct differences in the composition of peritoneal cells in *Gata6*-WT and *Gata6*-KO^{mye}. The composition of cells within the peritoneal cavity of *Gata6*-KO^{mye} has been reported to be markedly different, with a drastic reduction in the number of pMφs and a significant increase in the number of eosinophils and MHCII^{high}CD226⁺ DC-like cells compared to *Gata6*-WT mice (Rosas et al., 2014). Generally, *ex vivo* assays such as ELISAs were

performed using pMφs purified by adherence, termed APECs. Typically, this method has been reported to yield a population of cells consisting of approximately 90-95% pMφs. However, given the drastic differences in composition of peritoneal cells between the *Gata6*-WT and *Gata6*-KO^{mye}, it is possible that APECs from *Gata6*-KO^{mye} contain a population of cells that are a potent source of IL-6, which accounts for the significant increase in IL-6 detected by ELISA but not intracellular flow cytometry.

4.4.2 PTGIS is selectively expressed in pMφs and *GATA6*-dependent

Having established fundamental changes in the production of pro-inflammatory cytokines by *Gata6*-deficient pMφs, I sought to identify downstream mechanisms that may be responsible and modulate those to restore the inflammatory phenotype to that of pMφs from *Gata6*-WT mice. Microarray analysis revealed that one of the most down-regulated genes was *Ptgis*, the key enzyme required for PGI₂ synthesis. Historically, prostanoids have been regarded as pro-inflammatory mediators, but they are now widely recognised for their role in suppressing inflammation and immune responses. Prostanoids regulate the immune responses by modulating cytokine and chemokine production, migration, phagocytic potential, and cellular differentiation.

The profile of prostanoids produced is tissue-specific, which is attributed to the expression of prostanoid terminal synthases such as *Ptgis*. Therefore, the expression of terminal prostanoid synthases was analysed in pMφs from *Gata6*-WT and *Gata6*-KO^{mye}. Apart from the significant decrease in *Ptgis* expression, *Gata6*-deficient pMφs were found to have enhanced expression of *Tbxas1*, the enzyme responsible for the synthesis of TXA₂. Accordingly, analysis of prostanoid production in pMφs after stimulation with LPS showed that *Gata6*-deficient pMφs produced significantly less PGI₂ and more TXA₂ compared to pMφs from *Gata6*-WT mice (**Error! Reference source not found.**). PGI₂ and TXA₂ are best known for their prominent roles in the maintenance of vascular homeostasis. PGI₂ and TXA₂ have opposing roles and their imbalance has been implicated in various cardiovascular disorders and pulmonary hypertension (Tuder et al., 1999, Christman et al., 1992). Interestingly, there are reports which suggest that these opposing functions can be extended to inflammatory

responses, including the regulation of cytokine production. For instance, the inhibition of thromboxane synthase was shown to dramatically reduce TNF secretion by zymosan-stimulated human monocytes (Caughey et al., 1997). Conversely, PGI₂ analogues have been demonstrated to reduce the production of pro-inflammatory cytokines including TNF, IL-6 and IL-12 in BMDCs (Zhou et al., 2007). Interestingly, PGI₂ analogues promoted differential modulatory effects on IL-6 production depending on the specific cell type. Whereas the production of IL-6 in LPS-stimulated pMφs treated with PGI₂ analogues was greatly increased, alveolar Mφs did not increase the production of IL-6 to the same extent (Aronoff et al., 2007).

The production of prostanoids in pMφs after stimulation with TLR4 agonists or infection with *C. albicans* has been reported to predominantly consist of PGI₂ and PGE₂ (Suram et al., 2013, Norris et al., 2011). Furthermore, these prostanoids are produced rapidly, with significant amounts detected 30 minutes post stimulation (Suram et al., 2013). Therefore, the dramatic loss of PGI₂ and increase in TXB₂ production by LPS-stimulated *Gata6*-deficient pMφs may contribute to the dysregulated pro-inflammatory cytokine production.

4.4.3 Analogues restore ‘normal’ pro-inflammatory cytokine production in *Gata6*-deficient pMφs

Previous studies have strongly implicated PGE₂ as the critical prostanoid produced upon LPS stimulation that inhibits the production of TNF in pMφs (Katakami et al., 1988). However, many of these studies utilized elicited Mφs, which have very low *Ptgis* expression (Kuwamoto et al., 1997, Tripp et al., 1985, Wenzel et al., 1993). Furthermore, the prostanoid profile produced by resident pMφs and elicited Mφs has more recently been shown to be markedly different, which is likely to impart different effects on the inflammatory response (Norris et al., 2011). Indomethacin is an NSAID that targets COX1/2, thereby inhibiting the synthesis of downstream prostanoids. Interestingly, pMφs from *Gata6*-WT mice treated with indomethacin had significantly increased TNF but decreased IL-6 production compared to LPS alone (Figure 4.8). This suggests that prostanoids produced upon microbial recognition are critical regulators of cytokine production. Similarly, the production of TNF in F4/80^{high} pMφs from *Gata6*-KO^{mye} mice after treatment with indomethacin paralleled those of pMφs

from *Gata6*-WT mice. Conversely, the production of TNF and IL-6 production in LPS-stimulated F4/80^{low} pMφs treated with indomethacin was comparable to treatment with LPS alone, suggesting that the critical prostanoid regulating TNF and IL-6 production (in pMφs stimulated with LPS) is PGI₂, rather than PGE₂. However, this does not exclude that TXA₂ may contribute to cytokine regulation, which was found to be significantly higher in *Gata6*-deficient pMφs stimulated with LPS compared to pMφs from *Gata6*-WT mice. To address the contribution of PGI₂ in the regulation of TNF and IL-6 production, LPS-stimulated pMφs were treated with beraprost, a stable PGI₂ analogue. Beraprost significantly reduced TNF and increased IL-6 production in *Gata6*-deficient pMφs to levels comparable to pMφs from *Gata6*-WT mice treated with LPS alone. Beraprost treatment completely attenuated TNF production in pMφs from *Gata6*-WT and F4/80^{high} pMφs from *Gata6*-KO^{mye} mice, which is likely additive to the endogenous PGI₂ produced by these cells after LPS-stimulation. The use of PGI₂ analogues to investigate the function of native PGI₂ is contentious. Whilst endogenous PGI₂ has a high binding affinity to its cognate receptor and not for other prostanoid receptors, many PGI₂ analogues show equivalent binding to IP receptor and other prostanoid receptors including DP, EP, and, FP (Wise, 2003, Hata and Breyer, 2004, Wilson et al., 2011). Beraprost and cicaprost have been demonstrated to have higher binding affinity for IP receptor and are therefore considered better pharmacologic tools to investigate IP receptor-mediated biological responses (Tanaka et al., 2004). However, much of the ascribed functions of PGI₂ has been investigated using other analogues, which may occlude the function of native PGI₂. Furthermore, endogenous PGI₂ is unstable at physiological pH and has a very short half-life (< 2 minutes), rapidly forming the inactive 6-keto-PGF_{1α} (Smyth and FitzGerald, 2002, Lewis and Dollery, 1983). Conversely, the half-life of PGI₂ analogues is considerably longer, which will undoubtedly prolong its biological effects (Toda, 1988). Another limitation of PGI₂ analogues is that they do not compete for PGH₂, which may thereby alter the production of other prostanoids. Indeed, analysis of supernatants of LPS-stimulated pMφs showed a significant increase in TXB₂ in *Gata6*-deficient pMφs (Supplementary Figure 4**Error! Reference source not found.**).

Overall, these results indicate that the loss of endogenous PGI₂ production by LPS-stimulated *Gata6*-deficient pMφs leads to dysregulated cytokine production.

Furthermore, this effect is intrinsic to *Gata6*-deficient pMφs, as F4/80^{high} pMφs from *Gata6*-KO^{mye} mice responded similarly to those from *Gata6*-WT mice.

4.4.4 *Ptgis* transgene expression

To address the limitations of using PGI₂ analogues outlined above, the next aim was to reconstitute the expression of *Ptgis* in *Gata6*-deficient pMφs and evaluate whether this restored pro-inflammatory cytokine production to that of pMφs from *Gata6*-WT mice.

BMDMs do not express *Ptgis*, so were selected to validate the *Ptgis*-expressing lentiviral construct. The expression of the *Ptgis* transgene was greatly upregulated in BMDMs transduced with *Ptgis*-expressing lentivirus, as detected by qPCR and western blotting. Furthermore, upon LPS stimulation, BMDMs transduced with *Ptgis*-expressing lentivirus produced significantly more PGI₂ than BMDMs transduced with the *T2A-rCD2*. Furthermore, PGD₂, PGE₂ and TXB₂ were reduced in BMDMs transduced with *Ptgis*-expressing lentivirus. Since prostanoids are synthesised from the same common substrate PGH₂, the decrease in other prostanoids is likely because of increased competition for PGH₂, which has limited bioavailability. Having established that the *Ptgis* transgene could produce PGI₂, functionality was assessed. BMDMs that were non-transduced or transduced with either *T2A-rCD2* control or *Ptgis* were stimulated with LPS and the production of TNF and IL-6 determined by intracellular flow cytometry. Interestingly, BMDMs did not produce IL-6 in response to LPS after 3 or 6 hours stimulation with LPS (Figure 4.11 and Figure 4.12). However, BMDMs produced significant amounts of TNF when stimulated with LPS for either 3 or 6 hours but this was unaffected by *Ptgis* transgene expression (Figure 4.11 and Figure 4.12). Early prostanoid production in pMφs (0 -2 hours after stimulation) has been demonstrated to be dependent on COX-1, which is constitutively expressed in pMφs (Rouzer et al., 2004). Conversely, COX-2 is basally expressed at low levels but strongly induced by growth factors and pro-inflammatory stimuli including LPS, and is reported to be responsible for late prostanoid production (Barrios-Rodiles et al., 1999). It was noted that the quantity of prostanoids produced by BMDMs was considerably lower than that of pMφs (Norris et al., 2011). The expression of *Ptgs1* (COX-1) and *Ptgs2* (COX-2) have been reported to be substantially lower in BMDMs compared to peritoneal macrophages (von Moltke et al., 2012, Norris et al., 2011). Of

note, the expression of *Ptgs1* is over 1.5 fold down in *Gata6*-deficient pMφs compared to pMφs from *Gata6*-WT mice (Supplementary Figure 5). Furthermore, the induction of COX-2 expression after stimulation with LPS is also much lower in BMDMs compared to pMφs. Therefore, the *Ptgis* transgene may not be effective as the availability of PGH₂ (which is produced by the action of COX1/2) may be very low. Supporting this, LPS-stimulated BMDMs treated with indomethacin had comparable levels of TNF production compared to stimulation with LPS alone, suggesting that early endogenous prostanoids produced by BMDMs either don't regulate TNF production, or more likely that insufficient quantities of early prostanoids are produced by BMDMs because COX1/2 are not abundantly expressed, which are required for the generation of PGH₂. To exclude the possibility that BMDMs do not respond to PGI₂, LPS-stimulated BMDMs were treated with beraprost, which significantly reduced TNF production compared to LPS alone.

Resident pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice were transduced *in vivo* with T2A-*rCD2*- or *Ptgis*-expressing lentivirus, after which they were unstimulated or stimulated with LPS. Despite being injected with the same lentiviral preparation, the number of recoverable rCD2⁺ pMφs transduced with lentivirus was significantly reduced in *Gata6*-KO^{mye} mice compared to *Gata6*-WT mice (2.14% ± 0.04 and 19.85% ± 0.99 respectively).

4.4.5 Knockdown *Ptgis* in WT mice

To compliment the *Ptgis* reconstitution experiments in *Gata6*-deficient pMφs, shRNAs targeting *Ptgis* expression were designed and evaluated in C57BL/6 mice. The previous experiments showing that F4/80^{high} pMφs from *Gata6*-KO^{mye} mice had TNF and IL-6 production comparable to pMφs from *Gata6*-WT mice suggested the dysregulated production in *Gata6*-deficient pMφs was intrinsic. Given the relatively small population of F4/80^{high} pMφs within *Gata6*-KO^{mye}, the production of PGI₂ is unlikely to affect neighbouring F4/80^{low} cells, whereas autocrine and intracrine PGI₂ signalling is likely to modulate its own TNF and IL-6 production.

The production of TNF was not significantly different between pMφs transduced with shNSC- or sh*Ptgis*-expressing lentivirus. Furthermore, the production of TNF between transduced and non-transduced pMφs from the same mouse was comparable. Similarly, IL-6 production after 3 hours stimulation was not significantly different

between shNSC and sh*Ptgis*, and transduced and non-transduced cells. However, after 6 hours stimulation the production of IL-6 was significantly decreased in pMφs transduced with sh*Ptgis*.

However, there are a number of distinct differences between the non-transduced and *Ptgis*-transduced pMφs from the same mouse compared to F4/80^{high} and F4/80^{low} pMφs from the same mouse. Firstly, the relative percentage of F4/80^{high} is approximately 5%, whereas the non-transduced population is approximately 70-80% (in this experiment). The large population of non-transduced cells are likely to produce large quantities of PGI₂ that will likely saturate the culture medium and induce signalling in the transduced cells. Alternatively, the transduced population will not produce PGI₂ and will therefore mean the culture medium will have less total PGI₂ than pMφs transduced with shNSC.

4.5 Conclusion

Overall, *Gata6* controls the expression of *Ptgis*, the key enzyme that synthesises PGI₂. As such, the release of PGI₂ from *Gata6*-deficient pMφs following stimulation with LPS was significantly reduced compared to pMφs from *Gata6*-WT mice. Treatment of LPS-stimulated *Gata6*-deficient pMφs with a PGI₂ analogue restored the dysregulated production of TNF and IL-6, suggesting that PGI₂ may be a critical mediator released by pMφs to control inflammatory responses. Furthermore, the release of TNF from *Gata6*-deficient pMφs co-cultured with pMφs from *Gata6*-WT was comparable to monocultured pMφs from *Gata6*-WT mice. To establish whether PGI₂ is the critical mediator modulating inflammatory responses, *Gata6*-deficient pMφs were transduced with *Ptgis*-expressing lentivirus, which was technically challenging as the recovery of transduced *Gata6*-deficient pMφs was significantly lower than pMφs from *Gata6*-WT mice. Given the perturbed inflammatory responses of *Gata6*-deficient pMφs, it is possible that the poor recovery was due to increased cell death, or increased migration away from the peritoneal cavity and further experiments are needed to clarify the role of PGI₂ in mediating inflammatory responses. The design of shRNAs to knockdown *Ptgis* expression in *Gata6*-WT pMφs provided an alternative mechanism to study this, but poor transduction efficiency in these preliminary experiments has confounded conclusions regarding the role of PGI₂. Lentiviral

production protocols in our lab have since been optimised, which should enhance the transduction efficiency of future experiments.

Chapter Five

**Do other GATA factors regulate the
development of M ϕ s?**

5.1 Introduction

Langerhans cells (LCs) are resident myeloid cells of the epidermis, which were first described in 1868 by Paul Langerhans, a medical student who thought they were part of the nervous system due to their stellate processes. LC have long been considered the archetypal DC, based primarily on morphological features and their proficiency for antigen capture, processing, and subsequent migration to draining lymph nodes whereby they present antigen to naïve T cells via MHC molecules, reviewed by (Romani et al., 2010, Villadangos and Schnorrer, 2007). However, it has become increasingly evident, aided by cell tracing and transcriptional profiling studies that LCs exhibit specific developmental and homeostatic features, distinct from other DC populations (Hoeffel et al., 2012). The development of cDCs and pDCs is critically dependent on the cytokine fms-like tyrosine kinase 3 (Flt3) ligand (Flt3L) and its receptor (Flt3) (Waskow et al., 2008, Tussiwand et al., 2005, McKenna et al., 2000); knockout mice have a ten-fold reduction in pDCs and cDCs, whereas LC numbers were unaffected (Merad et al., 2008, Onai et al., 2007, Waskow et al., 2008). Similarly, GM-CSF is important for the development and differentiation of DCs, whereas GM-CSF-deficient mice have only a modest reduction of LCs *in vivo* (Kingston et al., 2009). In contrast, LC development is acutely dependent on CSF1R and transforming growth factor-beta 1 (TGF- β 1), exemplified by the lack of epidermal LCs in TGF- β 1- and CSF1R-deficient mice (Borkowski et al., 1996). The strict dependency of CSF1R for LC development is similar to tissue-resident macrophage populations, which are greatly diminished in CSFR1-deficient mice (Dai et al., 2002). However, the loss of CSF1, the cognate ligand for CSF1R, did not affect LC (Witmer-Pack et al., 1993, Takahashi et al., 1992) or microglia development (Ginhoux et al., 2006). IL-34, a stroma-derived cytokine was subsequently shown to bind to CSF-R1, suggesting that this alternative ligand may be responsible for the development of LCs and microglia (Lin et al., 2008). Accordingly, *IL-34*^{-/-} mice showed the same developmental defects as CSF-1R-deficient mice which were selectively deficient of LCs and microglia (Wang et al., 2012).

Another notable difference between cDCs/pDCs and LCs lies in their homeostasis and the control thereof. DCs are relatively short-lived and replenished throughout life from BM-precursors. Conversely, LCs are very long-lived and under steady state conditions are maintained throughout life by self-proliferation of LC or LC precursors seeded

during embryogenesis, independent of BM-derived precursor input (Hoeffel et al., 2012, Merad et al., 2002). Further studies suggest that a local pool of proliferating haematopoietic precursors seed the skin during embryonic development, which can give rise to LCs (Chang-Rodriguez et al., 2005). However, the development of LCs is differentially regulated in steady state and under inflammatory conditions. Following UV ablation of LCs, Gr-1^{high} monocytes were shown to be specifically recruited into the epidermis, where they proliferated locally and differentiated into LCs, a process is critically dependent on CSF1R (Ginhoux et al., 2006). Similarly, following UV irradiation, the existence of two distinct populations of LCs was reported, short-term and long-term LCs, which transiently or stably reconstitute the epidermis respectively (Seré et al., 2012). Short-term LCs are derived from Gr-1^{high} monocytes and are independent of the transcription factor Id2 (Seré et al., 2012, Chopin et al., 2013), which is critically required for the development of LCs and langerin⁺ dermal DCs (during ontogeny) (Hacker et al., 2003). Id2 is a critical mediator of TGF- β signalling, which is supported by an absence of LCs in both *ID2*- and *TGF- β* -deficient mice (Hacker et al., 2003). Conversely, the BM-precursors that give rise to long-term LCs are critically dependent on *Id2*. However, it is currently unclear whether long-term LCs contribute to the maintenance of LCs in the absence of an initial depletion of LCs, or whether LCs under steady state conditions are maintained exclusively by local self-proliferation of embryonically-seeded precursors.

Transcription factors play key roles in the development, maintenance, and function of myeloid cells. Recently, tissue-specific master regulators that control resident M ϕ s have been identified, which control the development and specify the transcriptional programs of tissue-resident M ϕ s, thereby enabling them to perform their niche-specific functions. For instance, the master regulator *SpiC* controls the development of red pulp M ϕ s, which phagocytose senescent erythrocytes and recycle heme-associated iron (Kohyama et al., 2009). Interestingly, physiologic stimuli from the niche in which tissue-resident M ϕ s reside can promote the expression of these master regulators. For instance, the expression of *Spic* is induced by heme, a metabolite of erythrocyte degradation (Halder et al., 2014). Similarly, retinoic acid in the peritoneal cavity induces the expression of the transcription factor *Gata6*, the central regulator of murine peritoneal M ϕ phenotype (Okabe and Medzhitov, 2014, Rosas et al., 2014, Gautier et al., 2014).

In addition to Id2, the transcription factor PU.1 has been shown to be important for steady state LC homeostasis. PU.1 controls LC differentiation through the induction of RUNX3, a critical component of the TGF- β signalling cascade in LCs (Fainaru et al., 2004). Interestingly, PU.1 synergizes with TGF- β to promote the expression of RUNX3. Accordingly, several master transcription factors have been shown to synergize with TGF- β , orchestrating the cell-type specific effects of TGF- β signalling (Mullen et al., 2011). Given the similarities between LCs and tissue-resident M ϕ s in their embryonic origin, capacity for self-renewal, longevity, and ligand-dependent development, we hypothesised that LCs are under the control of (tissue-specific) master regulators.

The GATA family of transcription factors are critical regulators for a diverse array of immune cells. Having identified Gata6 as the master regulator of murine peritoneal M ϕ function and phenotype, our research group is interested in how transcription factors control the development and function of other immune populations. Preliminary analysis of immune cell microarray datasets from the Immunological Genome Project (ImmGen) indicated that *Gata3* is selectively up-regulated in murine LCs. Furthermore, experiments in our lab performed by Dr Marcela Rosas showed *GATA3* was highly-expressed in LCs derived *in vitro* from the hematopoietic cell line MUTZ-3 compared to the precursor cells (Data unpublished).

The importance of GATA3 has been long established for developing and mature T cells, whereby it is required for the differentiation of T helper 2 cells, early T cell commitment CD4⁺ T cell development and β -selection (Zheng and Flavell, 1997). More recent studies have demonstrated that GATA3 is essential for the development and function of several immune subsets including the development, maintenance, and function of NK cells, NKT cells and ILC2 cells (Mjösberg et al., 2012, Samson et al., 2003, Kim et al., 2006).

Moreover, GATA3 is also expressed in many embryonic and adult tissues, including the kidneys, hair follicles, skin, and the central nervous system, in which several knockout and conditional knockout mouse models have demonstrated important functions (Kaufman et al., 2003, Grote et al., 2008). Given the role of GATA family members in the development, phenotype, and function of other immune cells, we

hypothesized that GATA3 may be important/synonymous in the LC-specific transcriptional program and the overarching function and phenotype thereof.

5.2 Chapter hypothesis and aims

The hypothesis tested in this chapter is that the development, maintenance, or effector functions of LCs are dependent on the transcription factor GATA3.

Aims

- To determine whether GATA3 is selectively expressed in human LCs
- To design and test the efficacy of lentiviral shRNA constructs on GATA3 knockdown
- To determine whether GATA3 knockdown effects LC differentiation
- To determine whether loss of GATA3 changes the phenotype of naïve and mature LCs

5.3 Results

5.3.1 GATA3 expression in murine and human LCs

To examine whether GATA family members are important for the development of myeloid cell subsets, publicly available microarray data sets were analysed for the expression of GATA family members in various murine immune cell populations (Figure 5.1A-C). The murine microarray expression data indicated that *GATA3* was selectively enriched in murine LCs, albeit at lower levels than T cell and NK cell subsets (Figure 5.1A-C). To determine whether GATA3 expression extends to human LCs, microarray expression data of human myeloid/DC populations from blood, skin, and *in vitro* derived DCs and LCs was analysed for GATA3 expression (Figure 5.1D). The expression of GATA3 was relatively low across all samples, but the expression was marginally higher in primary LCs and LCs derived *in vitro* from the MUTZ-3 cell line (MuLC) (Figure 5.1D).

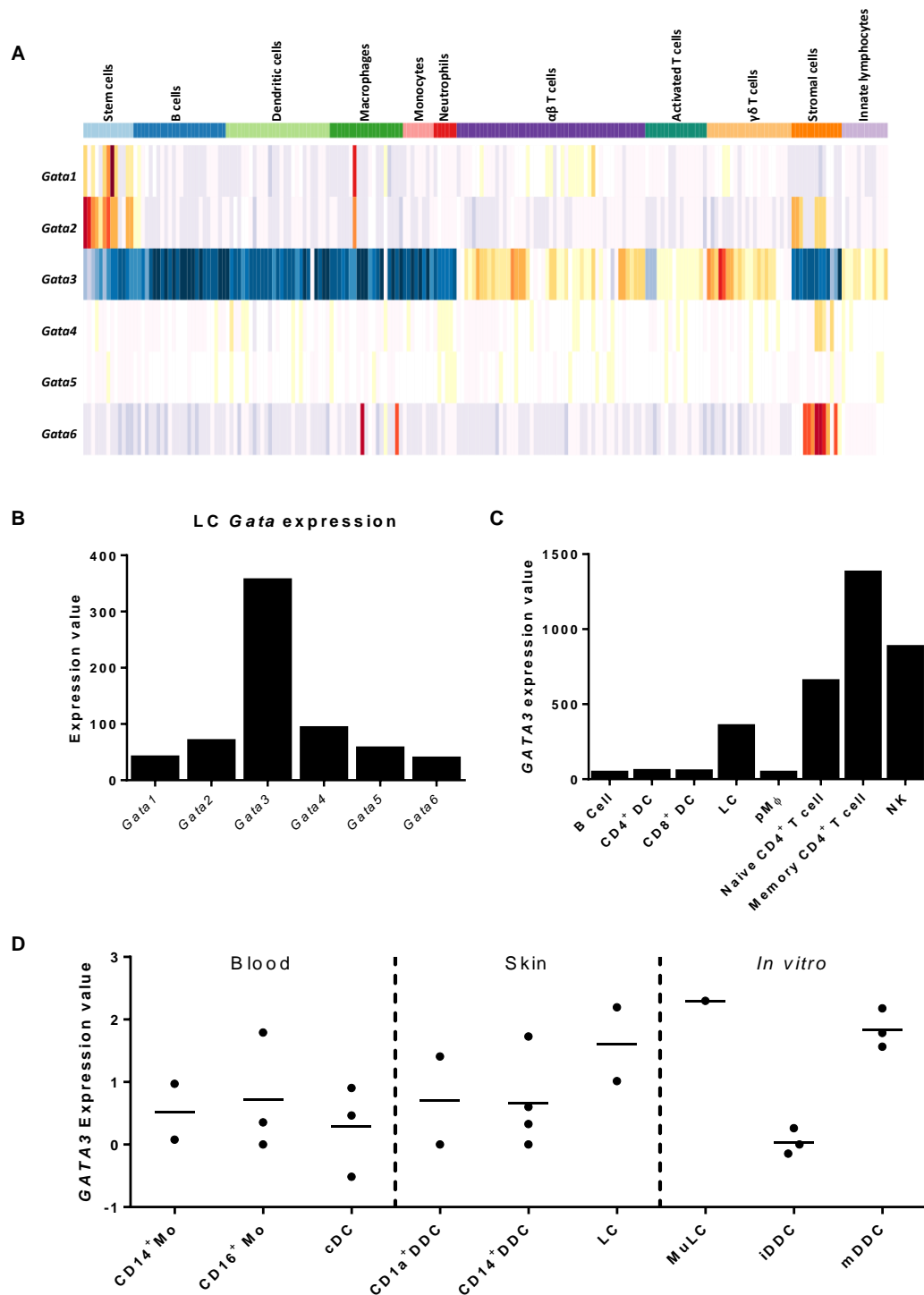


Figure 5.1 | Gata3 is selectively expressed in murine LCs.

(A-C) Microarray expression data across different murine immune cell populations. Data was assembled by the ImmGen consortium (Heng and Painter, 2008). (A) Heatmap showing the expression of *Gata* family members across different immune cell populations. (B) Expression values of *Gata* family members in murine LCs from Immgen consortium (C) The expression of *Gata3* in select immune cell populations, which unless stated otherwise were isolated from the spleen. (D) Quantile normalised expression of *GATA3* in human cells isolated from the blood, skin or generated *in vitro* from the MUTZ-3 cell line. Data was analysed from microarray data publicly available (GSE32400).

5.3.2 *In vitro* derived LCs upregulate GATA3 compared to precursors

To examine whether GATA3 is important for LC development, the human CD34⁺ acute myeloid leukaemia cell line MUTZ-3 was used as an *in vitro* model of LC development. MUTZ-3 cells can be differentiated into MUTZ-3-derived LCs (hereafter referred to as MuLCs) after 10 days of cytokine stimulation, and they exhibit similarities to primary LCs including the expression of langerin and the presence of Birbeck granules (Santegoets et al., 2006, Masterson et al., 2002). To evaluate whether the GATA3 is important for LC differentiation, the expression of GATA3 was determined in MUTZ-3 precursors and MuLCs by qPCR and flow cytometry respectively (Figure 5.2). The expression of *GATA3* was approximately 100-fold higher in MuLCs compared to MUTZ-3 precursors. However, no GATA3 protein was detectable by flow cytometry in CD1a⁺CD207⁺ MuLCs (Figure 5.2). The expression of *GATA3* was also determined in monocyte-derived LCs (MoLCs), but was found to be lower than both monocytes and monocyte-derived DCs (MoDCs).

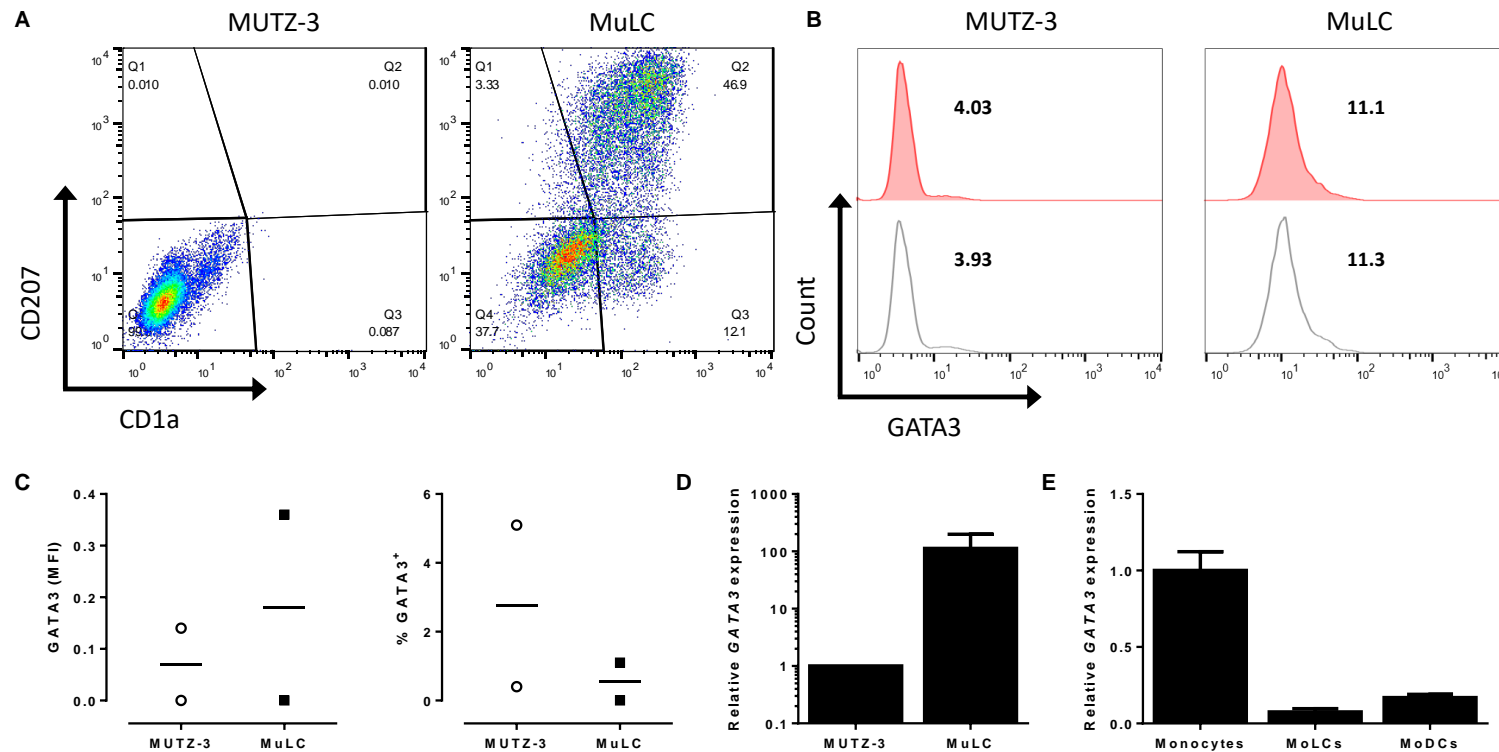


Figure 5.2 | MuLCs upregulate GATA3 compared to precursors, but no protein was detected.

(A) Representative bivariate plots showing the cell surface expression of CD1a and CD207 in MUTZ-3 precursors and LCs derived from MUTZ-3 (MuLCs). (B) Representative histogram showing isotype (empty histogram) and GATA3 (filled histogram) in MUTZ-3 precursors and CD1a⁺CD207⁺ MuLCs after 10 days of differentiation. The MFI is indicated on each plot. (C) Scatter plots showing GATA3 MFI (minus isotype control) and percentage positive cells from two independent experiments. (D) The expression of GATA3 was determined by qPCR relative to the endogenous control gene *YWHAZ* in MUTZ-3 precursors and CD1a⁺ MuLCs (MACS enriched). Data is mean \pm S.D from three independent experiments. (E) Bar chart shows the expression of GATA3 in monocytes, monocyte-derived LCs (MoLC) and monocyte derived DCs (MoDC) determined by qPCR relative to the endogenous control *YWHAZ*. Error bars indicate the 95% confidence interval of three technical replicates.

5.3.3 GATA3 protein is not detected during differentiation

The expression of *GATA3* detected by qPCR showed that it was highly-expressed in MuLCs compared to MUTZ-3 precursors. However, GATA3 protein was undetectable in MuLCs after 10 days of differentiation by cytokine stimulation (Figure 5.2). To evaluate whether GATA3 protein expression was transient, the expression of GATA3 in MUTZ-3 precursors undergoing differentiation at various time points was assessed by flow cytometry (Figure 5.3). The percentage of CD1a⁺CD207⁺ MuLCs on day 4 was similar to MUTZ-3 precursors, but steadily increased to approximately 8% by day 6 and over 50% by day 10 of differentiation. However, the expression of GATA3 was undetectable in MUTZ-3 precursors during differentiation at all time points analysed, in both CD207⁺ MuLCs and non-differentiated cells (Figure 5.3).

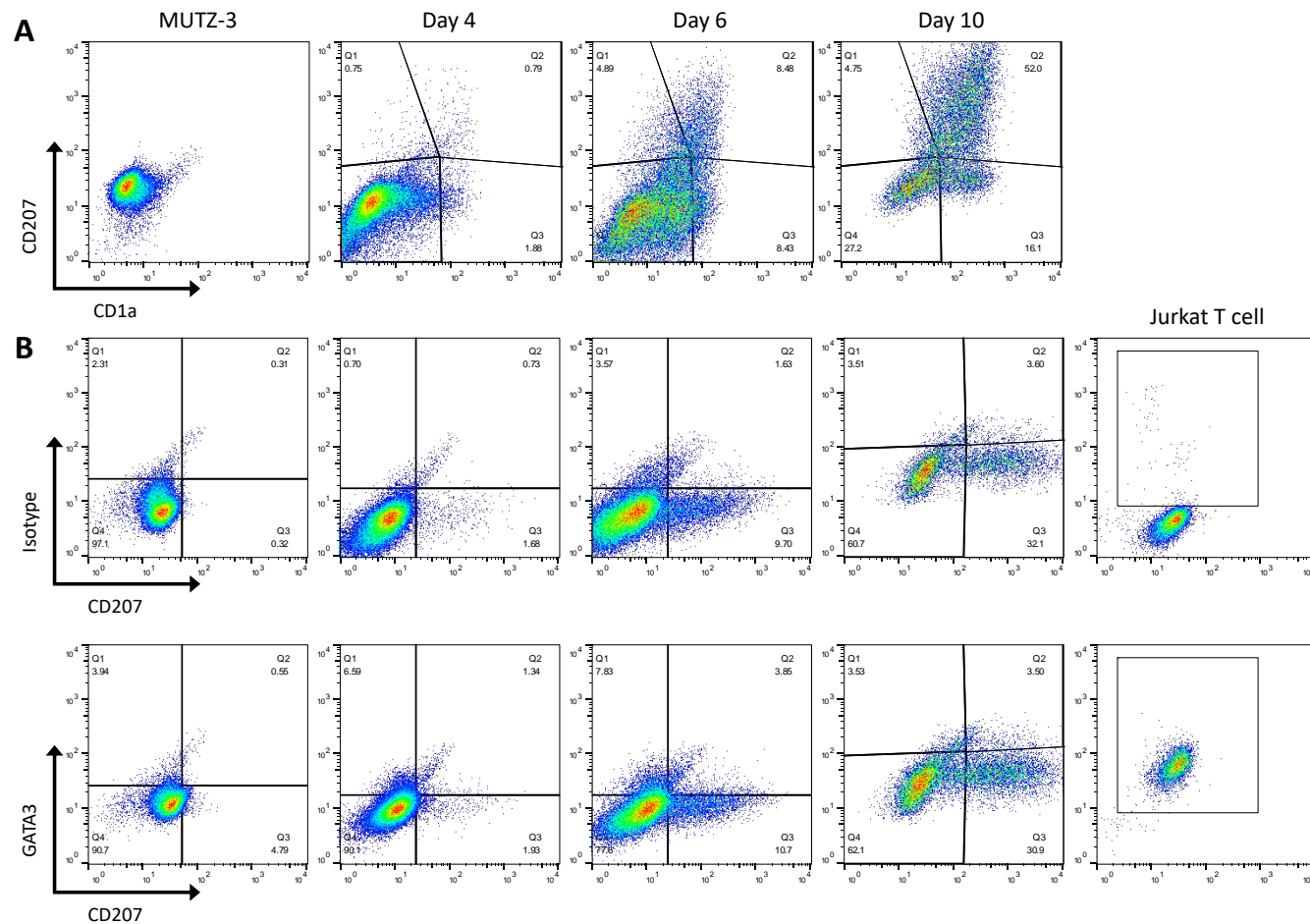


Figure 5.3 | The expression of GATA3 protein is undetectable in MuLCs during differentiation from MUTZ-3 precursors. (A, B) Bivariate plots showing the expression of (A) CD207 and CD1a and (B) isotype control or GATA3 in MUTZ-3 precursors and during their differentiation into MuLCs. Jurkat T cells were used as a positive control for GATA3 expression. $n=1$.

5.3.4 GATA3 protein is undetectable in mature MuLCs

GATA3 protein was undetectable in MUTZ-3 precursors at different stages of differentiation into MuLCs (Figure 5.3). GATA3 is critical for T helper type 2 (TH2) cell differentiation. Whilst IL-4 induces the expression of *GATA3* mRNA, the protein levels are insufficient to induce TH2 differentiation. Rather, simultaneous TCR-activation is required, which has been reported to stabilize GATA3 protein and enhance the rate of translation of GATA3 (Cook and Miller, 2010). Therefore, GATA3 protein levels in LCs may be dependent on specific extracellular stimuli that enhance its stability and translation. To determine whether GATA3 protein is detectable in MuLCs upon maturation, MuLCs were unstimulated or stimulated with LPS for 24 hours and the expression of GATA3 determined by flow cytometry. The expression of GATA3 was comparable, and effectively undetected in both unstimulated and LPS-stimulated MuLCs (Figure 5.4).

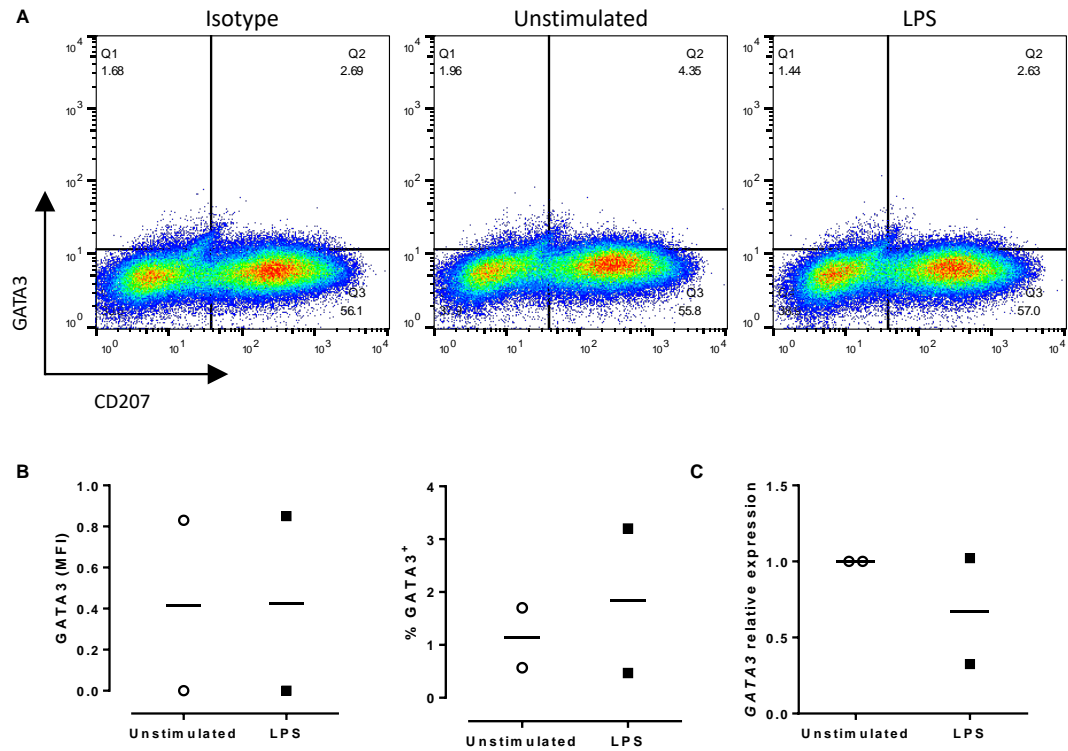


Figure 5.4 | The expression of GATA3 protein is undetectable in mature MuLCs. MuLCs were either unstimulated or stimulated with 1 $\mu\text{g ml}^{-1}$ LPS for 24 hours. **(A)** Representative bivariate plots showing CD207 and GATA3 expression in CD1a-enriched MuLCs. Experiment representative of two independent experiments. **(B)** Scatter plots show GATA3 MFI (minus isotype control) and percentage GATA3 positive MuLCs. Each point represents data from independent experiments. **(C)** The expression of GATA3 in CD1a-enriched MuLCs was determined relative to the endogenous control *YWHAZ*. Each point represents expression relative to unstimulated MuLCs from two independent experiments.

5.3.5 GATA3 shRNA design and efficacy

To investigate the role of GATA3 in LC development, maintenance, and function, lentiviral constructs expressing shRNA targeting GATA3 were designed. The efficacy of different shRNA constructs on the knockdown of GATA3 expression was determined in Jurkat T cells, which constitutively express GATA3. Jurkat T cells were transduced with two different lentiviral shRNA constructs targeting GATA3 and a non-silencing control (NSC) shRNA, and the expression of GATA3 determined by qPCR and flow cytometry (Figure 5.5).

The knockdown of *GATA3* expression in Jurkat T cells by shGATA-1 and shGATA-2 constructs was approximately 53% and 75% respectively (Figure 5.5 C). In addition to transcriptional analysis, flow cytometry was used to determine the knockdown of GATA3 expression, which showed that the transcriptional data strongly correlated flow cytometry analysis, in which shGATA3-1 was less efficacious in the knockdown of GATA3 compared to the shGATA3-2 construct, with a reduction in GATA3 expression of 45% and 70% respectively (Figure 5.5).

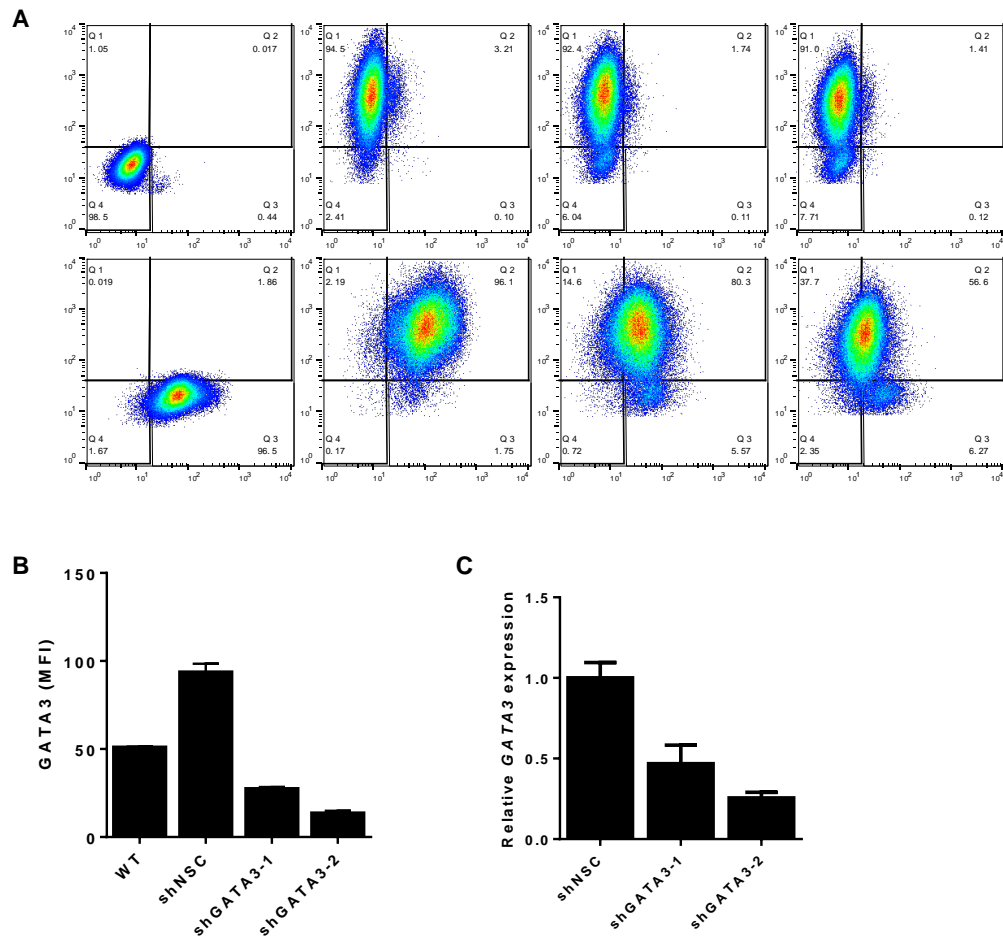


Figure 5.5 | shGATA3 design and efficacy.

Jurkat T cells were either non-transduced (WT) or transduced with lentiviruses encoding NSC, GATA3-1 or GATA3-2 shRNA constructs. **(A)** Representative bivariate plot showing GATA3 and rCD2 expression 7 days post-transduction. Data representative of two independent experiments **(B)** Bar graph shows mean GATA3 MFI (minus isotype control) \pm S.D from three biological replicates. **(C)** The expression of *GATA3* was determined by qPCR relative to the endogenous control gene *YWHAZ*. Error bars indicate the 95% confidence interval of three technical replicates.

5.3.6 GATA3 is not required for LC development.

Having established that the shRNA targeting GATA3 was efficacious, the next aim was to knock-down GATA3 expression in MUTZ-3 precursors to determine whether this would affect their differentiation into MuLCs. To determine whether GATA3 was important for the development of LCs, MUTZ-3 precursors were either non-transduced (WT) or transduced with lentiviral constructs expressing shNSC or shGATA3-2 (hereafter referred to as shGATA3) and subsequently differentiated into MuLCs. After 10 days, the differentiation of MUTZ-3 precursors was analysed by flow cytometry (Figure 5.6).

The percentage of CD1a⁺CD207⁺ LCs after 10 days differentiation from MUTZ-3 precursors was comparable between non-transduced and shNSC- or shGATA3-expressing cells ($p = 0.3488$ and $p = 0.7276$ respectively) (Figure 5.6). The expression of *GATA3* reduced by over 90% in MuLCs derived from precursors transduced with shGATA3 compared to non-transduced MuLCs. The expression of GATA3 was significantly reduced in MuLCs derived from precursors transduced with shGATA3 compared to those transduced with shNSC ($p = 0.0489$), confirming that MuLCs could differentiate without GATA3 (Figure 5.6B). The MFI of CD207 and CD1a was not significantly different between WT MuLCs and shNSC- or shGATA3-MuLCs (Figure 5.6E). However, the percentage of CD1a⁺CD207⁻ cells was significantly higher in precursors transduced with shNSC compared to non-transduced and shGATA3 cells ($p = 0.033$ and $p = 0.223$ respectively).

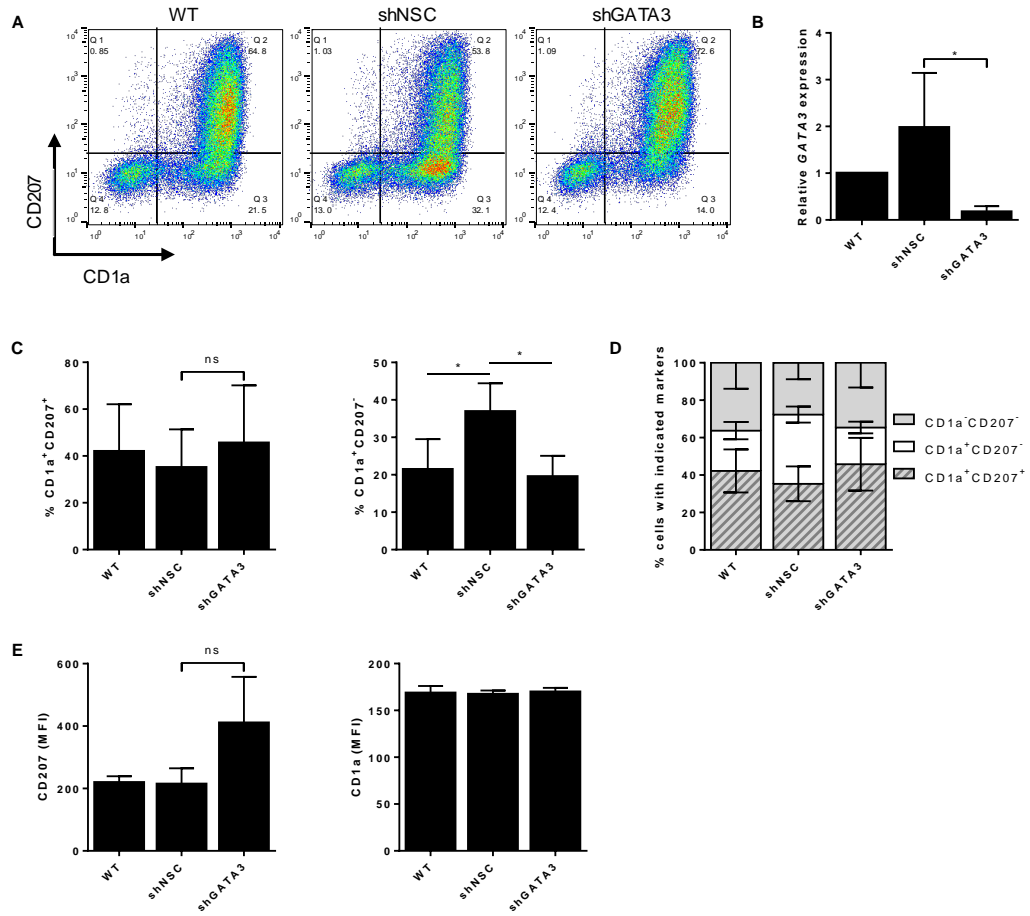


Figure 5.6 | Loss of GATA3 does not affect the development of MuLCs.

(A) Representative bivariate plots showing the cell surface expression of CD1a and CD207 in MuLC differentiated from MUTZ-3 precursors that were non-transduced (WT) or transduced with lentivirus expressing shNSC or shGATA3. (B) *GATA3* expression in CD1a⁺ MuLCs was determined by qPCR relative to the endogenous control *YWHAZ*. Data shows mean ± S.D from three independent experiments. (C) Bar chart shows percentage of CD1a⁺CD207⁺ and CD1a⁺CD207⁻ LCs after 10 days of cytokine differentiation. Data are presented as mean ± S.D of three independent experiments. (D) Stacked bar chart shows percentage of cells that were CD1a⁻CD207⁻, CD1a⁺CD207⁻ or CD1a⁺CD207⁺ after 10 days of cytokine differentiation. (E) Bar charts shows mean CD207 and CD1a MFI ± S.D after 10 days of cytokine differentiation. Statistical significance was determined by (B) a one-tailed paired t-test or (C, E) repeated measures one-way ANOVA with Tukeys multiple comparison post-test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, ns = not significant).

5.3.7 GATA3 is not required for LC receptor expression.

The loss of GATA3 had no significant effect on the differentiation of MUTZ-3 precursors to MuLCs (Figure 5.6). In addition to their role in the development of immune cells, transcription factors impart specific transcriptional control on mature cells that specifies distinct functional effector phenotypes. LCs express numerous cell surface receptors that enable them to recognise invading pathogens, and capture, process, and present antigen to naïve T cells.

The maturation of LCs has been functionally defined as the acquisition of potent immunogenic capacity (Banchereau and Steinman, 1998). This typically involves the upregulation of CD40, CD83, CD86 and HLA products, which are required for antigen presentation and co-stimulation of naïve T cells by LC. Therefore, to determine whether GATA3 is required for the maturation of LCs, cell surface receptor expression was determined by flow cytometry in unstimulated or LPS-stimulated MuLCs that were non-transduced (WT) or transduced with lentivirus expressing shNSC or shGATA (Figure 5.7). The percentage of cells expressing HLA-DR was comparable between MuLCs derived from precursors that were non-transduced or transduced with either shNSC or shGATA3 ($p = 0.9980$ and $p = 0.9094$ respectively) (Figure 5.7). Similarly, HLA-DR MFI was not significantly different ($p = 0.7730$ and 0.9079). Furthermore, stimulation with LPS did not significantly increase HLA-DR expression or percentage of positive cells. The percentage of cells expressing CD80 was not significantly different between WT MuLCs and shNSC- or shGATA3-MuLCs ($p = 0.8621$ and $p = 0.1819$). Similarly, CD80 MFI was comparable ($p = 0.8502$ and $p = 0.1041$). Stimulation with LPS did not significantly increase the percentage of CD80 positive cells or CD80 MFI. The percentage of cells expressing CD83 was comparable between WT MuLCs and shNSC- or shGATA3-MuLCs ($p > 0.9999$ and 0.9403). Likewise, CD83 MFI was comparable ($p > 0.9999$ and $p = 0.9999$). Stimulation with LPS did not significantly increase the percentage of CD83 positive cells or CD83 MFI.

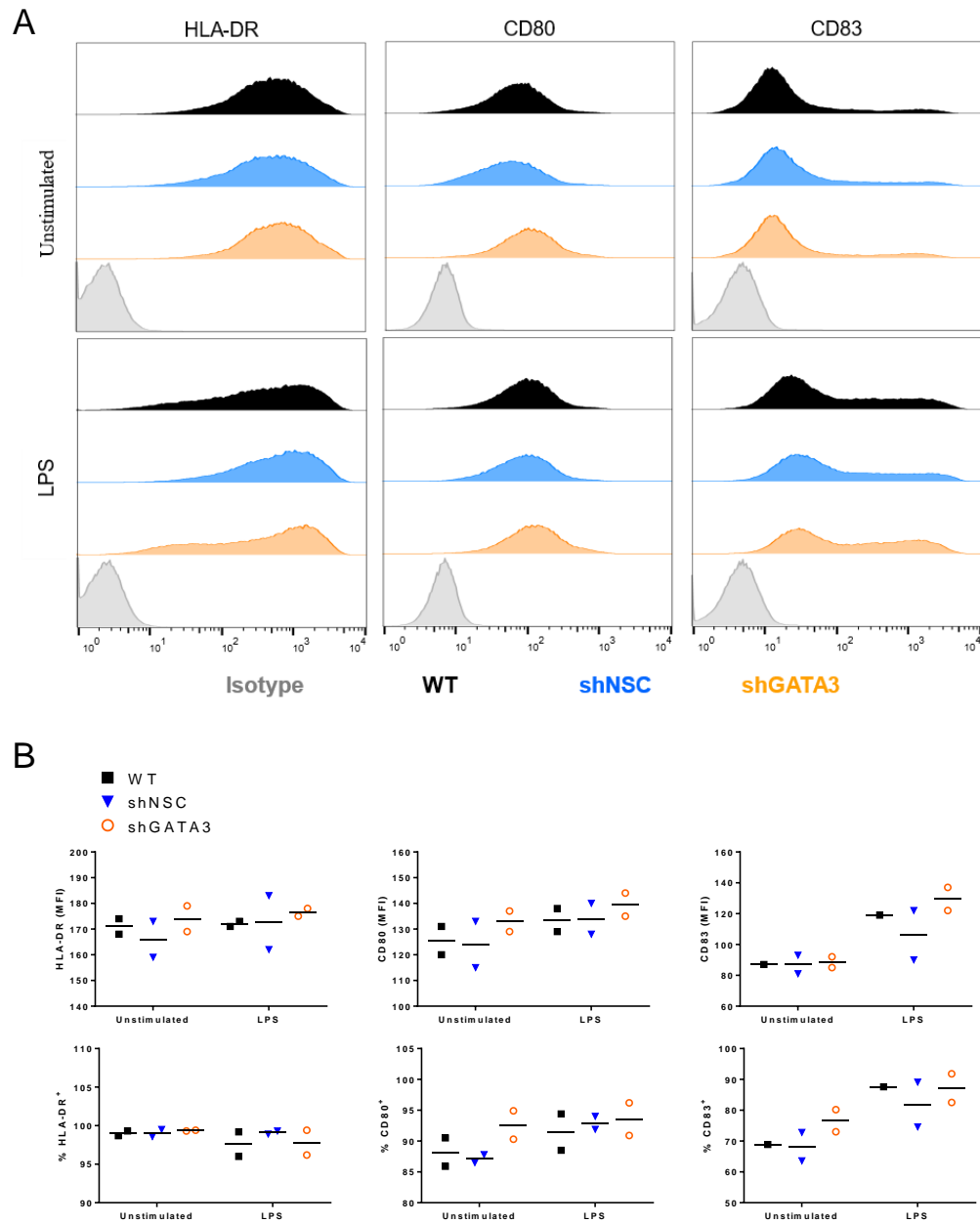


Figure 5.7 | LC cell surface receptor expression is unaffected by loss of GATA3. MuLCs were differentiated from MUTZ-3 precursors that were non-transduced or transduced with lentivirus expressing shNSC or shGATA. After differentiation, cells were either unstimulated or stimulated with $1 \mu\text{g ml}^{-1}$ LPS for 24 hours. (A) Cell surface receptor expression of CD1a-enriched MuLCs was determined by flow cytometry. (B) Scatter plots show MFI and percentage of cells expressing the indicated cell surface receptors. Each point represents data from independent experiments.

5.4 Discussion

5.4.1 GATA3 is up-regulated in mouse and human LCs

LCs are the tissue-resident myeloid cells of the epidermis that have long been described as the archetypal DC. However, unlike DCs which critically rely on Flt3/Flt3L and GM-CSF for their development, LC numbers are unaffected by deficiencies in either of these. Furthermore, DCs are short-lived and are derived from BM-precursors. Conversely, LCs have striking similarities with tissue-resident Mφs with respect to their embryonic origin, capacity for self-renewal, cytokine dependency, and their longevity (Romani et al., 2010).

Given the similarities between LCs and tissue-resident Mφs, I sought to identify whether LC development and function was dependent on a master regulator. Preliminary work within our research group indicated that GATA3 was enriched in LCs. Analysis of microarray expression data from different murine immune cell populations suggested that Gata3 was upregulated in LCs, although this was lower than in T cells and NK cells, for which the importance of Gata3 has already been ascribed. Analysis of human microarray data suggested that GATA3 may also be up-regulated in primary LCs and those derived from the MUTZ-3 cell line. However, the microarray dataset had a limited number of replicates, which restrained what conclusions could be made from this dataset. GATA family members are important for the development of many tissues. *Gata3*-deficient mice die at embryonic day 11, displaying severe abnormalities in the nervous system, aberrations in fetal liver haematopoiesis and massive internal bleeding (Pandolfi et al., 1995). GATA3 is expressed in both hematopoietic and non-hematopoietic tissues, including the central nervous system, the kidney, mammary glands, and of note for this study, skin (Grote et al., 2008, Kaufman et al., 2003, Kouros-Mehr et al., 2006).

5.4.2 GATA3 expression is up-regulated in MuLCs compared to MUTZ-3 precursors

To investigate whether GATA3 was important for LC development, the MUTZ-3 cell line was used as an *in vitro* source of LCs (MuLCs). LCs generated from MUTZ-3

precursors (MuLCs) have been shown to express langerin and contain Birbeck granules.(Santegoets et al., 2006). Furthermore, extensive phenotypic and functional comparative studies have suggested that MuLCs show a close resemblance to primary LCs (Santegoets et al., 2008). The expression of *GATA3* was highly up-regulated (~100 fold) in MuLCs compared to MUTZ-3 precursors, but no *GATA3* protein was detectable (Figure 5.2). The expression of *GATA3* in monocyte-derived LCs (MoLCs) was found to be ~10% of monocytes. The lack of detectable *GATA3* protein in MuLCs did not exclude its requirement for the development or function of LCs. Poor correlation of mRNA and protein levels have been reported, which are generally ascribed to translational control and the stability of the protein, yet these remain poorly understood (Maier et al., 2009). Genes that are involved in constitutive cellular processes such as translation (ribosomal proteins), respiration and metabolism (Glycolysis, TCA cycle) generally have stable mRNAs and proteins. Conversely, genes that have unstable mRNAs and proteins are enriched for transcription factors, chromatin modifying enzymes, and signalling genes (Schwanhauser et al., 2011). Thus, in general, critical regulators of dynamic processes such as transcription factors are under greater transcriptional and/or translational regulation than housekeeping genes. The ubiquitin-proteasome pathway is one of the major mechanisms for the degradation of short-lived regulatory proteins such as transcription factors. In CD4⁺ T cells, the TCR-mediated activation of the Ras-ERK-MAPK cascade regulates *GATA3* stability through the ubiquitin-proteasome pathway (Yamashita et al., 2005, Hosokawa et al., 2006, Shinnakasu et al., 2008). Further studies have reported that TCR activation leads to an mTORC-1-dependent increase in the rate of *GATA3* translation (Cook and Miller, 2010). Overall these studies show that the expression of master transcription factors in immune cells can be regulated at the level of translation and post-translation. Therefore, steady-state *GATA3* protein levels in LCs may be undetectable in fully-differentiated MuLCs, but transiently abundant during differentiation. To address this, the expression of *GATA3* protein was determined in MUTZ-precursors undergoing differentiation at various time points. However, no *GATA3* protein was detected at any of the time points analysed (Figure 5.3). Alternatively, like T cells which require TCR-activation for *GATA3* protein stabilization, LCs may depend on specific extracellular stimuli such as microbial ligands to stabilise *GATA3* protein levels. To address this, the expression of *GATA3* was determined in MuLCs unstimulated or stimulated with LPS, which induces the maturation of MuLCs. However, the

expression of GATA3 was undetectable in both unstimulated and LPS-stimulated LCs (Figure 5.4). Overall, despite *GATA3* mRNA being very highly expressed in MuLCs, GATA3 protein was undetectable in MuLCs both during the course of differentiation and upon maturation.

5.4.3 GATA3 is not required for LC development or maturation

To exclude the role of GATA3 in the development of LCs, lentiviral vectors expressing shRNAs targeting GATA3 were designed and the efficacy of GATA3 knockdown determined in Jurkat T cells, which abundantly express GATA3. Having determined the most efficacious shRNA construct, MUTZ-3 precursors were transduced and differentiated into MuLCs. The differentiation of MUTZ-3 into MuLCs was unaffected by the loss of GATA3 (Figure 5.6).

In addition to their role in the development of specific immune cells, tissue-specific transcription factors are required for specifying effector functions, through the induction of specific transcriptional programs. LCs predominantly reside in the epidermis where they are traditionally considered as the first line of defence against invading pathogens (Merad et al., 2008). More recently, the function of LCs has become more controversial, with some studies suggesting that their main function *in vivo* is immunoregulation rather than immune stimulation (Kaplan, 2010). LCs continuously probe their environment for invading pathogens by extending and retracting their dendrites. LCs express a plethora of PRRs including the C-type lectin receptors, which recognise carbohydrate moieties expressed by pathogens. Langerin (CD207) is a C-type lectin receptor found almost exclusively on LCs, with DDCs the only other cell type reported to express langerin (Ginhoux et al., 2007, Poulin et al., 2007, Bursch et al., 2007). The expression of both CD207 and CD1a, which are both highly expressed on LCs were unaffected by the loss of *GATA3* (Figure 5.6E).

The LC paradigm was largely established through studying LCs *in vitro*, which emphasized that LC have a unique capacity to induce/initiate primary immune responses. In peripheral tissues, they acquire antigens and subsequently migrate to lymph nodes where they present processed peptides to naïve T cells (Romani et al.,

2010, Villadangos and Schnorrer, 2007). Upon microbial stimulation, MuLCs (and primary LCs) are described in the literature as having ‘matured’. Upon ‘maturation’, LCs up-regulate the expression of genes such as those encoding the co-receptors CD80, CD40. GATA3-deficiency did not affect the expression of these markers, suggesting that GATA3 is not required for the maturation of MuLCs.

5.4.4 Conclusion

The expression of *GATA3* was highly upregulated in MuLCs compared to their MUTZ-3 precursors. Together with public microarray data, the expression of *GATA3* appeared to be selectively upregulated. Despite the high expression of *GATA3*, no detectable protein was present, either in differentiating MuLCs or in fully-differentiated MuLCs. Furthermore, GATA3 was not required for the differentiation of MuLCs, nor was it required for their maturation. Based on these observations, I conclude that GATA3 is not required for the development, maintenance, or maturation of LCs from the MUTZ-3 cell line.

Chapter Six

General Discussion

6.1 Introduction

Tissue-resident macrophages are a highly heterogeneous cell type, present in virtually every mammalian tissue, where they play a fundamental role in development, homeostasis, tissue repair and host defence responses (Davies et al., 2013, Gordon and Taylor, 2005). Tissue-resident macrophages were long thought to be derived from BM precursors, although recent lineage-tracing studies challenged this long-held view and indicate that embryonic precursors give rise to many tissue-resident macrophage populations. In adulthood, different tissue-resident populations have differential requirements for BM-derived replenishment, but most consist of a mixture of embryonically-derived and monocyte-derived macrophages. Comprehensive transcriptional analysis of resident macrophage populations suggested macrophage populations from different organs exhibited considerable transcriptional diversity, as would be predicted from their phenotypic diversity (Gautier et al., 2012), indicating that environmental cues may condition different tissue-resident macrophage populations to perform their tissue-specific roles. Recent evidence suggests that distinct tissue-specific master regulators govern the phenotypic diversity and specialised functions of different tissue-specific macrophages. Our research group as well as other groups identified the transcription factor *Gata6* as a critical regulator of a peritoneal macrophage-specific transcriptional program (Okabe and Medzhitov, 2014, Gautier et al., 2014, Rosas et al., 2014). Interestingly, retinoic acid derived from peritoneal omental tissue was shown to drive induce the expression of *Gata6* (Okabe and Medzhitov, 2014), suggesting that environmental factors have a critical role in specifying the tissue-specific functions of different tissue-resident macrophage populations. Analogous factors have been identified in other tissue-resident macrophage populations, such as *Spi-C*, which is essential for the development of splenic red pulp macrophages (Kohyama et al., 2009). However, genetic deletion of these tissue-specific transcriptional regulators typically results in the ablation of the specific macrophage populations, for instance the loss of splenic red pulp macrophages in mice deficient of *Spi-C*, and absence of metallophilic and marginal zone macrophages in mice deficient of *LXR α* (A-Gonzalez et al., 2013, Kohyama et al., 2009). Conversely, genetic deletion of *Gata6* in macrophages does not completely ablate the population of peritoneal macrophages, albeit it leaves them at reduced numbers. Thus, *Gata6*-deficient pM ϕ s provide a unique tool to investigate how tissue-

specific transcription factors regulate macrophage function during homeostasis and in response to environmental stimuli, such as those encountered during inflammation.

Lineage-specific transcription factors, such as PU.1, which is essential for the development of macrophages, are thought to co-ordinately regulate macrophage identity and function in tandem with tissue-specific transcription factors during the steady state. Furthermore, recent evidence suggests that the accessibility of macrophage-specific transcriptional regulatory elements (enhancers and promoters) is imparted by tissue- and lineage-specific transcription factors (Lavin et al., 2014, Gosselin et al., 2014). As such, the ability of stimulus-induced transcription factors such as NF- κ B to elicit gene expression changes is thought to be dictated by the accessibility of enhancers and promoters specified by tissue- and lineage-specific transcription factors. However, it is largely unclear how transcriptional master regulators control tissue-specific macrophage phenotype during the initiation of the inflammatory response and the resolution thereof.

Aims

To date, limited studies have addressed how the loss of these tissue-specific transcriptional factors effects the inflammatory responses of tissue-resident macrophage populations. As such, the work presented in this thesis aimed to identify whether Gata6 was important for the regulation of the inflammatory response and its resolution, and whether analogous factors control the development of other tissue-resident macrophages.

6.2 Gata6 regulates inflammatory responses of peritoneal macrophages

Recent studies have identified a number of tissue-specific transcription factors that control the development and function of tissue-resident macrophage populations. However, our current understanding of how these transcription factors regulate inflammatory responses is unclear. To address this, pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were stimulated with an array of TLR ligands, as well as fungal ligands and the production of pro-inflammatory cytokines determined.

6.2.1 Dysregulated TNF and IL-6 production in LPS-stimulated *Gata6*-deficient pMφs

The production of TNF from LPS-stimulated pMφs from *Gata6*-WT mice was transient, with peak production detected at 3 hours and undetectable in culture supernatants at 12 hours. Conversely, LPS-stimulated *Gata6*-deficient pMφs produced significantly more TNF at 3 hours, which was sustained and detectable after 24 hours (Figure 4.3). Flow cytometric cytokine staining supported the significant increase in peak TNF production following stimulation with LPS for 3 hours. However, the production of IL-6 detected by flow cytometry was significantly lower in *Gata6*-deficient pMφs compared to those from *Gata6*-WT mice, whereas IL-6 detected by ELISA was comparable. These discrepancies may result from distinct differences in the composition of peritoneal cells in *Gata6*-WT and *Gata6*-KO^{mye}, as *ex vivo* assays such as ELISAs were performed using pMφs purified by adherence, termed APECs. Thus, it is possible that APECs from *Gata6*-KO^{mye} contain a population of cells that are potent sources of IL-6. Alternatively, soluble factors released through the classical secretion pathway (inhibited by GolgiPlug™ in flow cytometric cytokine production assays) may modulate the production of IL-6. Preliminary experiments of MACS-enriched pMφs from *Gata6*-WT and *Gata6*-KO^{mye} suggest that the production of IL-6 is comparable at early and late time points, and the production of TNF and IL-1β reflect the other experimental observations in this thesis using APECs.

6.2.2 *Gata6* regulates the activation of the NLRP3 inflammasome

The production and release of IL-1 β from macrophages is tightly controlled through the combination of two distinct signals: transcription as well as maturation and release, commonly referred to as the two-signal model for IL-1 β processing and release (Martinon et al., 2002). In macrophages, LPS stimulation alone fails to induce robust processing and release of mature IL-1 β (Franchi et al., 2012). Strikingly, *Gata6*-deficient pM ϕ s released mature IL-1 β and IL-18 upon stimulation with LPS alone, whereas minimal IL-1 β and IL-18 were detected in supernatants from LPS-stimulated pM ϕ s from *Gata6*-WT mice (Figure 3.1). The production of IL-18 from LPS-stimulated *Gata6*-deficient pM ϕ s was significantly lower than that of IL-1 β , which may reflect the differences in the intracellular pool of IL-18 and IL-1 β . Supporting this, *Gata6*-deficient pM ϕ s primed with LPS and stimulated with nigericin secreted significantly more IL-1 β than IL-18 (65 ng ml⁻¹ and 1.5 ng ml⁻¹ respectively). Inhibition of caspase-1 or NLRP3 inflammasome activation significantly reduced the production of IL-1 β from LPS-stimulated *Gata6*-deficient pM ϕ s. Although the release of mature IL-1 β in response to LPS alone ('Priming' signal) has been well described for murine BMDCs and human monocytes (Gaidt et al., 2016, He et al., 2013), secretion from macrophages is generally strictly dependent on two distinct signals. Conversely, genetic defects that lead to spontaneous IL-1 β secretion by macrophages have been reported. BMDMs deficient of all three inhibitor of apoptosis proteins (cIAP1, cIAP2, and XIAP) exhibited spontaneous NLRP3 inflammasome activity and IL-1 β secretion in response to LPS alone (Vince et al., 2012). Furthermore, another study suggested that BMDMs deficient of XIAP alone secreted IL-1 β in response to LPS alone (Yabal et al., 2014). Similarly, ablation of *Atg16l1* resulted in IL-1 β secretion in response to LPS stimulation alone (Saitoh et al., 2008). More recently, LPS-stimulated BMDMs deficient of *Tnfrsf3* (which encodes the NF- κ B inhibitor A20) were shown to spontaneously secrete IL-1 β (Duong et al., 2015). However, the release of mature IL-1 β from LPS-stimulated *Gata6*-deficient pM ϕ s is distinct from these previous studies. Firstly, macrophages deficient of all IAP proteins, A20 or *Atg16l1* primed with LPS and activated with ATP secreted significantly more IL-1 β compared to wild-type macrophages, suggesting that these factors control the magnitude of NLRP3 activation and subsequent IL-1 β processing. Conversely, *Gata6*-

deficient pMφs primed with LPS and stimulated with soluble NLRP3 stimuli (ATP and nigericin) responded normally, secreting comparable levels of IL-1β as pMφs from *Gata6*-WT mice (Figure 3.5), suggesting that *Gata6* does not have a prominent role in regulating the magnitude of NLRP3 activation. Another distinguishing feature is the direct or indirect specificity of LPS to induce the secretion of IL-1β from *Gata6*-deficient pMφs, but not any of the other TLR ligands tested. However, for the studies carried out in this thesis, a dose-response curve for IL-1β and IL-18 release from *Gata6*-WT and *Gata6*-KO^{mye} pMφs was only performed with the TLR4 ligand LPS. As such, it is possible that the concentrations of TLR ligands used to stimulate pMφs from *Gata6*-WT and *Gata6*-KO^{mye} pMφs were sub-optimal. As such, it is possible that the release of IL-1β and IL-18 from *Gata6*-deficient pMφs may not be restricted to LPS stimulation alone and that the failure of other TLR ligands to induce the release of IL-1β and TNF from *Gata6*-KO^{mye} is an experimental artefact. Conversely, a range of TLR ligands have been reported to induce IL-1β secretion from macrophages deficient of all IAP proteins or A20 secreted mature IL-1β in response to a range of TLR ligands.

Having established that the secretion of mature IL-1β from LPS-stimulated *Gata6*-deficient pMφs, the underlying molecular mechanisms of NLRP3 inflammasome activation were investigated. A wide array of exogenous and endogenous stimuli have been reported to activate the NLRP3 inflammasome (Latz et al., 2013). Given the structural and molecular diversity of these stimuli, it is likely that they all bind directly to the NLRP3 inflammasome, as such, it has been suggested that inflammasomes most likely monitor cellular homeostasis which is perturbed by activatory stimuli. In addition to their role in metabolism, mitochondria are complex regulators of cellular homeostasis. Perturbation of ROS, intracellular K⁺ concentration or lysosomal damage have been reported to induce mitochondrial dysfunction (Tschopp, 2011), which are also implicated in the activation of the NLRP3 inflammasome. As such, mitochondrial dysfunction has been strongly linked to the activation of the NLRP3 inflammasome, predominantly through the generation of mtROS and the release of mtDNA and cardiolipin (Nakahira et al., 2011, Zhou et al., 2011, Iyer et al., 2013). However, the role of mitochondria in the activation of the NLRP3 inflammasome is contentious (Lawlor and Vince, 2014), which could reflect cell-type and stimulus-dependent divergence in the requirement for mitochondria in the activation of the inflammasome.

Naïve *Gata6*-deficient pMφs had enhanced mtROS compared to pMφs from *Gata6*-WT mice. During OXPHOS, the leakage of electrons at complex I and III leads to the generation of superoxide, which is typically dismutated to hydrogen peroxide by the action of superoxide dismutase (SOD)-1 and -2. However, high levels of mtROS are implicated in the activation of the inflammasome. Consistent with increased mtROS, *Gata6*-deficient pMφs had a higher basal rate of OXPHOS than pMφs from *Gata6*-WT mice (Figure 3.7). The release of IL-1β was significantly reduced in LPS-stimulated *Gata6*-deficient pMφs treated with the mitochondria-targeted antioxidant, MitoTEMPO, compared to LPS-stimulation alone. Dysfunctional mitochondria are ordinarily targeted and removed for degradation in autophagosomes, in a process termed mitophagy (Lazarou et al., 2015). Indeed, perturbations of mitophagy have been strongly linked to inflammasome activation and IL-1β release (Zhong et al., 2016). The accumulation of dysfunctional mitochondria (non-respiring, as determined by MitoTracker Deep Red staining) was higher in both unstimulated and LPS-stimulated *Gata6*-deficient pMφs compared to those from *Gata6*-WT mice, suggesting that they may have defective mitophagy which can perpetuate the production of mtROS and release of mtDNA, which can thereby activate the NLRP3 inflammasome.

6.3 Wild-type macrophages restore inflammatory responses of *Gata6*-deficient macrophages

The penetrance of Cre-mediated excision of *Gata6* in peritoneal macrophages is approximately 95%, resulting in a *Gata6*⁺ population of pMφs within *Gata6*-deficient mice. *Gata6*-deficient pMφs from young mice can be identified by flow cytometry by Tim4⁺F4/80^{low}, whereas the population of *Gata6*⁺ pMφs within the *Gata6*-KO^{mye} mice exhibit Tim4⁺F4/80^{high} staining, comparable to pMφs from *Gata6*-WT mice (Rosas et al., 2014). Intriguingly, the production of TNF and IL-6 in LPS-stimulated *Gata6*⁺ pMφs from *Gata6*-KO^{mye} mice was comparable to pMφs from *Gata6*-WT mice, whereas the Tim4⁺F4/80^{low}*Gata6*⁻ pMφs exhibited greatly enhanced TNF and reduced IL-6 production (Figure 4.5). These results suggest that the aberrant production of these cytokines is cell intrinsic, and that contact-dependent mechanisms and/or soluble factors released (such as cytokines, lipids, or DAMPs) from *Gata6*-deficient pMφs have no effect on the inflammatory responses of *Gata6*⁺ pMφs.

Conversely, co-cultured pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice (1:1 culture ratio) had significantly reduced secretion of TNF and IL-1β compared to monocultured *Gata6*-deficient pMφs (Figure 3.14 and Figure 4.6), suggesting that contact-dependent mechanisms and/or the release of soluble factors from pMφs from *Gata6*-WT mice modulate the inflammatory responses of *Gata6*-deficient pMφs. Given the relatively small population of *Gata6*⁺ pMφs in *Gata6*-KO^{mye} mice, it is perhaps not surprising that they are insufficient to significantly regulate the inflammatory responses of neighbouring *Gata6*-deficient pMφs in monoculture experiments. Overall, these observations suggest that *Gata6* expression regulates either contact-dependent mechanisms and/or the release of soluble factors that dampen inflammatory responses, rather than DAMPs released by *Gata6*-deficient pMφs exacerbating inflammatory responses of neighbouring cells.

6.4 Soluble factors

Having established that *Gata6*-deficient pMφs have markedly perturbed inflammatory responses and that co-cultured pMφs from *Gata6*-WT mice could restore the aberrant release of IL-1β and TNF, attention was directed to the identification of mechanisms by which *Gata6* regulates the inflammatory response and resolution thereof.

6.4.1 Cytokine and purinogenic signalling

The differential response of *Gata6*-deficient pMφs to different TLR ligands provided an insight into possible mechanisms underlying the release of IL-1β from *Gata6*-deficient pMφs stimulated with LPS but not any of the other TLR ligands tested. One of the most striking observations was that similar to the release of IL-1β, the prolonged release of TNF was restricted to stimulation with LPS (Figure 4.3). On the basis of these observations, I hypothesised that IL-1β released from LPS-stimulated *Gata6*-deficient pMφs was mediated through the enhanced and sustained release of TNF. To address this, TNF signalling was inhibited in LPS-stimulated pMφs with etanercept. Whilst etanercept significantly reduced the release of IL-1β by over 30%, it did not completely prevent the release of IL-1β (Figure 3.15). TNF signalling in macrophages can lead to the activation of NF-κB, which induces the expression of genes including *Il1b* and *Tnf* (Yarilina et al., 2008). Accordingly, TNF signalling may enhance the transcription of *Il1b*, increasing the pool of pro-IL-1β in *Gata6*-deficient pMφs, which can be subsequently processed and released. Thus, inhibition of TNF signalling might reduce the expression of pro-IL-1β, but not directly contribute to the activation of the inflammasome. Furthermore, the release of IL-1β from *Gata6*-deficient pMφs stimulated with recombinant TNF was comparable to unstimulated pMφs. However, the responses of pMφs to co-stimulation with LPS and recombinant TNF were not investigated, which may synergize and result in the aberrant release of IL-1β. Similarly, LPS-stimulated pMφs treated with rIL-1Ra (which blocks autocrine and/or paracrine IL-1β signalling) had no significant effects on the production of either IL-1β or TNF compared to LPS stimulation alone.

Our group has previously reported that CD73 expression is greatly diminished in *Gata6*-deficient pMφs (Rosas et al., 2014). This ectoenzyme along with CD39 is highly expressed in resident peritoneal macrophages, which mediate the conversion of extracellular ATP into adenosine (Zimmermann and Braun, 1999, Kaczmarek et al.,

1996). Macrophages release ATP into the extracellular milieu following exposure to TLR ligands and its subsequent processing to adenosine is reported to suppress the production of proinflammatory cytokines including TNF, whilst enhancing the production of anti-inflammatory cytokines such as IL-10 (Cohen et al., 2013). As such, the release of ATP and the subsequent generation of adenosine represents a possible mechanism through which resident macrophages resolve inflammatory responses. As *Gata6*-deficient pMφs have significantly reduced CD73 expression, I hypothesised that they would have a deficiency of adenosine generation, which is a potent anti-inflammatory mediator that can down-regulate pro-inflammatory cytokine expression and up-regulate the expression of anti-inflammatory cytokines. The release of IL-10 was significantly lower in LPS-stimulated *Gata6*-deficient pMφs compared to those from *Gata6*-WT mice (Figure 3.16). Interestingly, LPS-stimulated pMφs treated with adenosine had significantly reduced TNF production but the secretion of IL-1β was unaffected, further supporting a limited role for TNF signalling in the release of IL-1β. However, the addition of exogenous adenosine might not reflect the concentration of endogenous adenosine produced from the hydrolysis of ATP. In support of this, attempts to inhibit CD73 did not enhance the production of TNF or IL-1β in LPS-stimulated pMφs from *Gata6*-WT or *Gata6*-KO^{mye} mice.

6.4.2 *Gata6* regulates inflammatory responses through eicosanoid production

One of the most down-regulated genes in *Gata6*-deficient pMφs was the gene encoding prostacyclin synthase, a terminal prostanoid synthase that catalyses the generation of prostacyclin (PGI₂) from prostaglandin H₂. Previous studies have shown pMφs predominantly produce PGI₂, PGE₂, and LTC₄ after stimulation with LPS, TNF, and IL-1 as well as *C. albicans*- and zymosan (Brock et al., 1999, Norris et al., 2011, Suram et al., 2013). PGI₂ analogues have been shown to reduce the production of pro-inflammatory cytokines including TNF, IL-6, and IL-12 in BMDCs (Zhou et al., 2007). More recently, PGI₂ and PGE₂ analogues were reported to enhance the transcription of anti-inflammatory mediators and attenuate the secretion of TNF from pMφs stimulated with *C. albicans* (Suram et al., 2013). LPS-stimulated *Gata6*-deficient pMφs had significantly reduced production of both PGI₂ and PGE₂ and increased TXB₂ (the stable metabolite of TXA₂) compared to pMφs from *Gata6*-WT mice (Supplementary Figure 4).

To examine whether the dysregulated production of TNF and IL-6 were a result of significantly reduced PGI₂, LPS-stimulated pMφs from *Gata6*-KO^{mye} were treated with beraprost, a PGI₂ analogue. Similar to previous studies, beraprost significantly reduced the production of TNF in *Gata6*-deficient pMφs which was comparable to TNF production in LPS-stimulated pMφs from *Gata6*-WT mice. Conversely, the production of TNF by LPS-stimulated *Gata6*-deficient pMφs was unaffected when treated with the COX1/2 inhibitor indomethacin, whereas TNF production in LPS-stimulated pMφs from *Gata6*-WT mice were significantly enhanced. Similarly, beraprost treatment significantly reduced the release of IL-1β (60% reduction) from LPS-stimulated *Gata6*-deficient pMφs (Figure 3.6). Given that the reduction of IL-1β by beraprost treatment is more pronounced than that of etanercept, it is unlikely that the suppression of IL-1β is solely a consequence of reduced TNF production. PGI₂ analogues have been reported to decrease the expression of pro-inflammatory cytokines including IL-1β and TNF, mediated through the elevation of intracellular cAMP, which culminates in the inhibition of NF-κB signalling. Additionally, cAMP has been suggested to modulate NLRP3 inflammasome activation through direct binding to NLRP3 (Lee et al., 2012). Thus, cAMP produced downstream of PGI₂ can potentially downregulate IL-1β by suppressing the transcription of *Il1b*, and through the direct inhibition of NLRP3 inflammasome activation. However, treatment with DB or RP (a cAMP analogue or an inhibitor of the activation of cAMP-dependent protein kinases by cAMP, respectively) did not significantly affect the release of IL-1β from LPS-stimulated *Gata6*-deficient pMφs (Figure 3.6).

Gata6-deficient pMφs stimulated with LPS secreted significantly more TXB₂, less PGE₂, and barely detectable levels of PGI₂ compared to pMφs from *Gata6*-WT mice (Supplementary Figure 4). TXB₂ has been typically ascribed pro-inflammatory functions and has been reported to enhance TNF production by zymosan-stimulated human monocytes (Caughey et al., 1997). However, indomethacin treatment did not reduce the production of TNF from *Gata6*-deficient pMφs stimulated with LPS, suggesting that the increase in TXB₂ does not enhance TNF signalling in this context. PGE₂ has generally been described as having anti-inflammatory effects, but further study is needed to determine whether endogenous PGE₂ produced by LPS-stimulated *Gata6*-deficient pMφs suppressed IL-1β release. Many of the ascribed functions of PGI₂ have been established in studies utilising PGI₂ analogues (Suram et al., 2013).

However, the use of these occlude the function of endogenous PGI₂ in several ways. Firstly, many of the PGI₂ analogues used in the literature (treprostinil and iloprost) exhibit comparably high binding affinity for IP receptor as other prostanoid receptors including DP₁, EP₁, and EP₂ (Whittle et al., 2012, Hata and Breyer, 2004). In contrast, the PGI₂ analogue beraprost is reported to have a much lower affinity for other prostanoid receptors and is therefore considered better pharmacologic tools to investigate IP receptor-mediated biological responses (Tanaka et al., 2004). Additionally, whilst the half-life of endogenous PGI₂ is very short (< 2 minutes), the half-life of analogues is considerably longer, which undoubtedly prolong its biological effects and likely don't reflect the function of endogenous PGI₂.

To investigate how endogenous PGI₂ effected the production of pro-inflammatory cytokines, the gene encoding *Ptgis* was cloned into lentiviral constructs with the aim of reconstituting *Ptgis* expression in *Gata6*-deficient pMφs and determine whether restoring endogenous PGI₂ production would restore pro-inflammatory cytokine production to that of pMφs from *Gata6*-WT mice. BMDMs transduced with the *Ptgis*-expressing lentivirus simulated with LPS failed to modulate the production of TNF at both 3 and 6 hours. This is likely because the expression of *Ptgs1* and *Ptgs2* (which encode COX-1 and COX-2 respectively) was significantly lower in BMDMs than pMφs, which are required for the generation of PGH₂, the universal substrate for downstream prostanoid synthases (von Moltke et al., 2012, Norris et al., 2011). Indeed, LPS-stimulated BMDMs treated with indomethacin had comparable production of TNF to LPS treatment alone, suggesting that endogenous eicosanoids are not produced at these early time points by these cells. The transduction of *Gata6*-deficient pMφs transduced *in vivo* with *Ptgis*-expressing transgene was significantly lower than those from *Gata6*-WT mice, which may result from increased migration away from the peritoneal cavity (such as to the omentum) or increased cell death. As such, the production of TNF from *Ptgis*-expressing pMφs was comparable to the control lentivirus. Given the small population of positive *Ptgis*-expressing *Gata6*-deficient pMφs, it is not surprising that the production of TNF was not significantly different, as the production of PGI₂ from this population is likely not enough to significant effect the larger number of non-transduced cells, synonymous with the *Gata6*⁺ population in *Gata6*-KO^{mye} being unable to modulate the inflammatory responses of the predominant Tim4⁺F4/80^{low}*Gata6*⁻ population.

To overcome the technical issues in transducing *Gata6*-deficient pMφs, I designed shRNA constructs targeting *Ptgis* with the aim recapitulating the aberrant production of TNF and IL-6 of LPS-stimulated *Gata6*-deficient pMφs. The production of TNF was comparable at both 3 hours and 6 hours between the cells transduced with shNSC (control virus) and shPtgis in wild-type pMφs. Similarly, IL-6 production was comparable at 3 hours in transduced cells expressing shNSC and shPtgis lentiviral constructs. However, the production of IL-6 in LPS-stimulated pMφs after 6 hours was significantly lower in pMφs transduced with shPtgis compared to shNSC (Figure 4.16). However, the production of IL-6 from the non-transduced and shPtgis-transduced pMφs was not significantly different, which could suggest that PGI₂ produced from the transduced population are modulating the production of IL-6 in the non-transduced population. However, the transduction efficiency in these experiments was particularly low (~5%), which probably confounded the effect of *Ptgis*-knockdown. Our lentiviral production protocols have since been optimised in our group. Thus, further experimentation with a higher percentage of transduced pMφs may help to delineate the role of PGI₂ in TNF and IL-6 production from LPS-stimulated pMφs.

6.5 LPS stimulation induces cell death in *Gata6*-deficient pMφs

The release of mature IL-1 β from macrophages is reported to be strictly dependent on pyroptosis, which is mediated by the caspase-1 or caspase-11-dependent cleavage of gasdermin D, which leads to the formation of membrane pores culminating in the loss of plasma membrane integrity and release of IL-1 β (He et al., 2015, Shi et al., 2015, Kayagaki et al., 2015). A similar study suggested that all NLRP3 activatory stimuli induce necrosis, which was proposed to be the convergence point of various stimuli for the activation of the NLRP3 inflammasome activation and subsequent processing and release of IL-1 β (Cullen et al., 2015). However, this study preceded the identification of the role of gasdermin D in mediating pyroptosis, so it is likely that the necrotic cell death reported in this study is pyroptosis mediated by gasdermin D cleavage.

To investigate whether the release of mature IL-1 β from LPS-stimulated *Gata6*-deficient pMφs was associated with an increase in cell death, unstimulated and LPS-stimulated pMφs were stained with propidium iodide (PI⁺), which is excluded by viable cells. The percentage of PI⁺ pMφs was significantly higher in LPS-stimulated *Gata6*-deficient pMφs, compared to unstimulated *Gata6*-deficient pMφs and both unstimulated and LPS-stimulated pMφs from *Gata6*-WT mice (Figure 3.8).

The percentage of PI⁺ pMφs is relatively small compared to that from pMφs primed with LPS and stimulated with either ATP or nigericin, which is consistent with the comparatively smaller release of IL-1 β from LPS-stimulated *Gata6*-deficient pMφs (typically 1 – 2 ng ml⁻¹) compared to release from LPS-primed stimulated with either ATP or nigericin (35 and 65 ng ml⁻¹ respectively). Surprisingly, inhibition of either caspase-1 or NLRP3 inflammasome activation failed to attenuate LPS-induced cell death in *Gata6*-deficient pMφs, but did prevent the release of mature IL-1 β (Figure 3.4 and Figure 3.9). Furthermore, the inhibitors exhibited no apparent toxicity in unstimulated *Gata6*-deficient pMφs. Apart from caspase-1, pyroptosis has been reported to be mediated by the non-canonical inflammasome activation, mediated by caspase-11 (Kayagaki et al., 2015). Caspase-11 was recently identified to directly recognise and bind intracellular LPS (Hagar et al., 2013), and has been implicated in

protection against intracellular Gram-negative bacteria in the cytosol that have escaped vacuoles (Aachoui et al., 2013). Caspase-11 mediates the activation of the NLRP3 inflammasome which in turn leads to the activation of caspase-1 and processing and release of IL-1 β . Interestingly, caspase-11 has been shown to directly cleave gasdermin d and subsequent pyroptosis independently of caspase-1 activation. As such, it is possible that cell death in *Gata6*-deficient pM ϕ s stimulated with LPS is mediated by caspase-11, which accounts for the NLRP3- and caspase-1-dependent release of IL-1 β and independent cell death. However, further study is needed to establish whether gasdermin D is cleaved in LPS-stimulated *Gata6*-deficient pM ϕ s, which would implicate pyroptosis as the mechanism of cell death. If LPS-stimulated *Gata6*-deficient pM ϕ s undergo pyroptosis, inhibition of caspase-11 (or shRNA knockdown) may address the underlying mechanism driving pyroptosis.

Interestingly, the percentage of PI⁺ *Gata6*-deficient pM ϕ s stimulated with Pam3CSK4 was comparable to pM ϕ s from *Gata6*-WT mice, suggesting that the underlying mechanisms regulating the release of IL-1 β may also induce cell death. TNF signalling can also induce pro-apoptotic and RIP3-dependent necroptosis signalling cascades in macrophages, culminating in the activation of the inflammasome and IL-1 β secretion in macrophages deficient of XIAP (Yabal et al., 2014). However, etanercept treatment did not attenuate cell death in LPS-stimulated *Gata6*-deficient pM ϕ s (Figure 3.15), suggesting that TNF signalling does not contribute to LPS-induced cell death in *Gata6*-deficient pM ϕ s. Supporting this, preliminary experiments suggest that treatment of LPS-stimulated *Gata6*-deficient pM ϕ s with beraprost does not significantly reduce cell death, despite reducing the production of both TNF and IL-1 β (Figure 3.6 and Figure 4.8).

Alternatively, LPS stimulation may induce cell death and the release of IL-1 β by independent mechanisms. Dramatic reduction of peritoneal macrophages in *Gata6*-KO^{mye} mice has been attributed to increased apoptosis under homeostatic conditions (Gautier et al., 2014), whereas another study attributed the reduction to increased accumulation of pM ϕ s in the omentum (Okabe and Medzhitov, 2014). As such, LPS-stimulation may induce apoptotic cell death in *Gata6*-deficient pM ϕ s as opposed to pyroptotic cell death. *Gata6*-deficient pM ϕ s stimulated with LPS had significantly more caspase-3⁺ pM ϕ s than unstimulated pM ϕ s and both unstimulated and LPS-stimulated pM ϕ s from *Gata6*-WT mice (Figure 3.11). In contrast to the observations

of Gautier and colleagues, the percentage of caspase-3⁺ naïve pMφs from *Gata6*-deficient pMφs was not significantly different from pMφs from *Gata6*-WT mice, which was supported by comparable annexin V staining (Figure 3.10). The increase in apoptosis of *Gata6*-deficient pMφs was attributed to the marked reduction in *Aspa* expression, although it is noteworthy that approximately 10% of pMφs from *Aspa*-deficient mice were apoptotic, compared to over 20% reported for *Gata6*-deficient pMφs (Gautier et al., 2014). Furthermore, apoptotic cells *in vivo* are typically cleared rapidly, unless the phagocytes have an impairment in the phagocytosis of apoptotic cells (Taylor et al., 2000, O'Brien et al., 2006, Elliott and Ravichandran, 2016). Similarly, apoptotic cells are normally cleared *in vitro* with similarly rapid dynamics. Impaired clearance of apoptotic cells typically results in the accumulation of late apoptotic and secondary necrotic cells (PI⁺), which are thought to release endogenous danger signals that can exacerbate inflammatory responses, which has been implicated in the development of autoimmune diseases such as systemic lupus erythematosus (Kaplan, 2004, Elliott and Ravichandran, 2016). As caspase-3⁺ *Gata6*-deficient pMφs are readily detectable after stimulation with LPS, it may suggest that *Gata6*-deficient pMφs have impaired apoptotic clearance, which would be supported by the increase in PI⁺ pMφs after stimulation with LPS, which may simply reflect secondary necrotic cells.

Strikingly, the percentage of PI⁺ *Gata6*-deficient pMφs was significantly reduced when co-cultured with pMφs from *Gata6*-WT mice (Figure 3.14). The results from these experiments suggest that either *Gata6*-WT pMφs attenuate cell death through contact-dependent mechanisms and/or the release of soluble factors, or that *Gata6*-WT pMφs phagocytose early apoptotic *Gata6*-deficient pMφs, which prevents the progression of apoptotic cells to secondary necrotic cells. The clearance of apoptotic cells is thought to be predominantly non-phlogistic, and is characterised by the suppression of pro-inflammatory cytokine production and the increased production and release of pro-inflammatory mediators such as TGF-β and IL-10 (Fadok et al., 1998, Meagher et al., 1992). Furthermore, secondary necrotic cells may release endogenous signals, which can subsequently activate the inflammasome. One of the limitations of these experiments is they are predominantly end-point assays, which occlude the dynamics of apoptotic induction, as such, future experiments utilising real-

time monitoring of caspase-3/7 activation may provide critical insights into the kinetics of caspase-3 activation.

6.6 Do other GATA factors regulate the development of Mφs?

The final hypothesis tested was that the development, maintenance, or effector functions of LCs are dependent on the transcription factor GATA3. LC development is strictly dependent on the transcription factor *Id2* for their development (Chopin et al., 2013, Hacker et al., 2003). Microarray expression data from human and murine suggested that GATA3 was selectively up-regulated. The CD34⁺ MUTZ-3 cell line provides a valuable tool for the generation of *in vitro* human LCs, which share similar characteristics to primary LCs (Masterson et al., 2002, Santegoets et al., 2008, Santegoets et al., 2006). Consistent with public microarray data, *GATA3* was highly expressed in MuLCs, whereas it was barely detectable in MUTZ-3 precursors. However, flow cytometric analysis of GATA3 showed that it was comparable between the precursors and MuLCs (and barely detectable). I hypothesized that GATA3 may be required for the differentiation of precursors into MuLCs and thus not-detectable in differentiated MuLCs. However, flow cytometric staining for GATA3 was comparable over the course of differentiation. Supporting this, the knockdown of *GATA3* had no significant effect on the differentiation of precursors to MuLCs, despite the significant reduction in *GATA3* expression (Figure 5.2). The high expression of *GATA3* but no detectable protein may indicate that stimuli might be required for the transcription of *GATA3*. To test this, the expression of GATA3 was determined in naïve MuLCs and those matured through stimulation with LPS. However, GATA3 was undetectable and in both naïve and mature MuLCs, and knockdown of GATA3 did not affect the maturation of MuLCs, as determined by comparable expression of maturation markers and key LC genes. Based on these observations, I conclude that GATA3 is not required for the development, maintenance, or maturation of MUTZ-3 derived LCs. It will be interesting to analyse GATA3 expression in both primary mouse and human cells, which could be determined by immunofluorescent microscopy for GATA3 in skin biopsies.

6.7 Conclusions

The work in this thesis establishes the importance of tissue-specific transcription factors on the control of macrophage inflammatory responses, which to date, is poorly understood. The mechanisms underlying the differential requirement of one- or two-signals for the processing and release of IL-1 β is poorly understood. This is the first report of LPS-stimulation alone inducing the release of mature IL-1 β in macrophages deficient of a tissue-specific transcription factor, suggesting that tissue-specific transcription factors controlling tissue-resident macrophages exert tight regulatory control over the release of IL-1 family cytokines and other pro-inflammatory mediators including TNF and NO, which could have important indications for the field of macrophage biology and the regulation of inflammatory responses.

6.8 Future perspectives

RNA-sequencing of unstimulated and LPS-stimulated pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} will provide valuable information that may help to identify perturbed pathways that contribute to the abnormal pro-inflammatory cytokine production exhibited by *Gata6*-deficient pM ϕ s. Tissue-specific transcription factors can regulate enhancer landscapes, which in turn make the regulatory elements of genes more accessible to stimulus-dependent transcription factors such as NF- κ B, induced downstream, of LPS signalling. The current data of unstimulated pM ϕ s from *Gata6*-WT and *Gata6*-KO^{mye} mice therefore this data doesn't provide an accurate picture of the genes that are dysregulated during inflammation in *Gata6*-deficient pM ϕ s. Understanding this may aid in the identification of pathways that are contributing to the phenotypes shown in this thesis.

The restoration of normal inflammatory responses of *Gata6*-deficient pM ϕ s when co-cultured with pM ϕ s from *Gata6*-WT mice is striking. To determine whether soluble mediators such as cytokines mediate this restoration, further study should perform these co-culture experiments in trans well tissue culture plates, which will allow any soluble mediators to exert their effects. Similarly, the transfer of conditioned medium generated from LPS-stimulated *Gata6*-WT pM ϕ s to those from *Gata6*-KO mice should support any observations from the trans well studies. Following on from these studies,

BMDMs deficient of any candidate genes could be co-cultured with pMφs from *Gata6*-KO mice.

Currently, our understanding of the control of macrophage function during homeostasis and inflammation is limited. One of the pertinent questions to be resolved is whether BM-derived tissue-resident macrophages can fully adopt the phenotype and function of the embryonically-derived Mφs they replace. Tissue-derived signals such as retinoic acid in the peritoneum induces the expression of *Gata6*, which in turn specifies the function and phenotype of peritoneal macrophages. Furthermore, more recent studies have shown that tissue-specific and lineage-specific transcription factors co-ordinately regulate enhancer landscapes in tissue-resident macrophages (Gosselin et al., 2014, Lavin et al., 2014), which in turn regulates the accessibility of regulatory elements to stimulus-dependent transcription factors, such as NF-κB. However, whether origin and ontogeny impacts the regulation of enhancer landscapes and subsequent gene expression of BM-derived compared to embryonically-derived tissue-resident macrophages remains to be addressed. Single-cell sequencing of embryonically-derived and BM-derived tissue-resident macrophage populations should greatly aid our understanding of how their origin and ontogeny effects their function and phenotype. Interestingly, different tissue-resident macrophage populations have differential requirements for BM-derived tissue-resident macrophage input. Furthermore, the requirement of BM-derived input has been reported to be highly gender specific for peritoneal macrophages (Bain et al., 2014), although the underlying mechanisms governing this are yet to be determined. Interestingly, this study showed that BM-derived tissue-resident pMφs did not express the phagocytic receptor Tim4, which is highly expressed on embryonic-derived pMφs. This might indicate that BM-derived pMφs may not acquire the complete transcriptional profile of the embryonic cells they gradually replace, which may impact the function of pMφs. However, extensive transcriptional studies are needed to fully define whether BM-derived pMφs can adopt the phenotype of embryonically-derived pMφs. Interestingly, another study showed that BM-precursors transplanted into irradiated mice acquired 90% of the transcriptional profile of their embryonic-derived peritoneal and alveolar macrophages (Lavin et al., 2014), although this was tissue-specific, as BM-derived Kupffer cells acquired less than 50% of the tissue-specific enhancers of their embryonic counterparts. Similarly, Bruttger *et al.* showed that BM-derived microglia

that colonize irradiated brains (microglial depletion) had over 2, 000 genes that were differentially expressed compared to embryonic microglia (Bruttger et al., 2015). Collectively, these results suggest that either a substantial component of the transcriptional profile of microglia and Kupffer cells are determined by their embryonic origin, or alternatively, that irradiation disrupts brain and liver homeostasis, preventing normal imprinting of BM-derived cells. Consistent with the latter, upon conditional deletion of Kupffer cells, Scott and colleagues showed that BM-derived Kupffer cells acquired an almost identical transcriptional profile to their embryonic counterparts (Scott et al., 2016). Likewise, when lungs were protected from irradiation in BM chimeric animals, BM-derived alveolar macrophages were reported to have almost identical transcriptional profile as embryonic-derived alveolar macrophages (Gibbins et al., 2015). Collectively, these studies suggest that disruption of tissue-homeostasis can have critical implications for the phenotype and function of tissue-resident macrophage function. This is particularly relevant in an inflammatory context, in which a plethora of stimuli (cytokines, microbial ligands, DAMPs) can potentially dictate tissue programming and ultimately affect the function of tissue-resident macrophage populations. One recent study of zymosan-induced peritonitis reported that BM-derived pMφs (that populated the peritoneum following inflammation) persisted in the peritoneum for at least 2 months post-resolution (Newson et al., 2014), congruent with the persistence of BM-derived pMφs up to 2 months after thioglycolate-induced peritonitis (Yona et al., 2013). Furthermore, the phenotype of the resident pMφs after 60 days post-zymosan was different to naïve pMφs, suggesting that inflammatory contexts can re-programme tissue resident macrophage populations, which can ultimately affect their functional characteristics. Overall, the function and phenotype of tissue-resident macrophages following inflammation remains poorly characterised and further study is needed.

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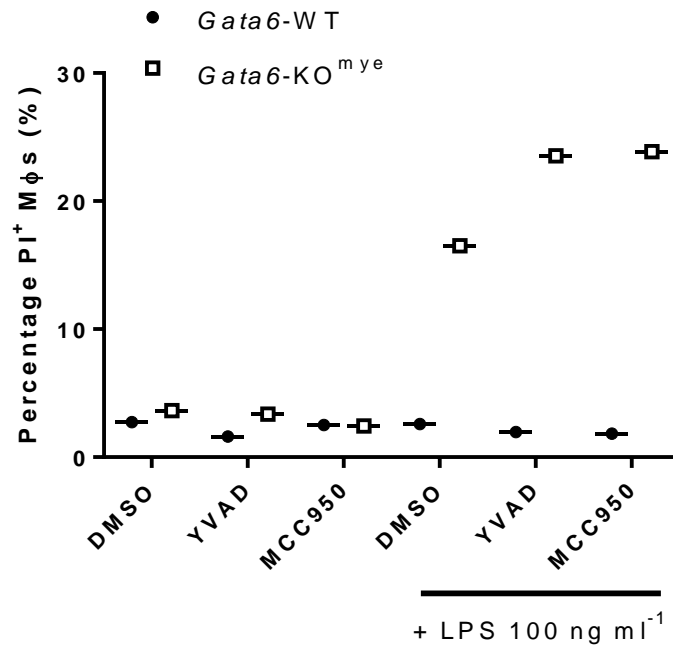
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Appendices

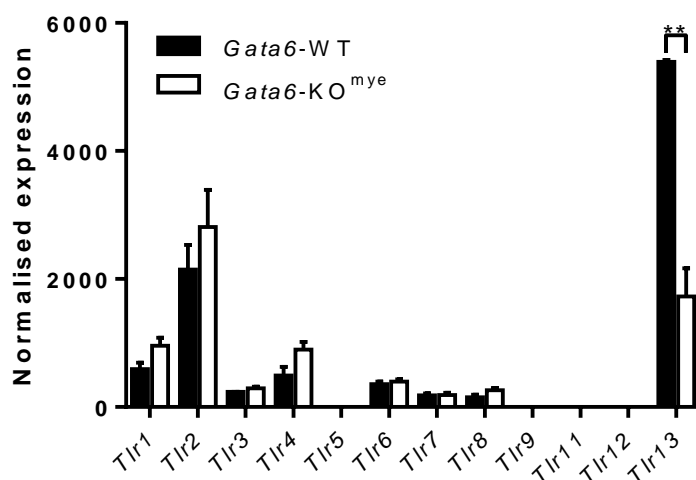
Appendix A | Caspase-1 or NLRP3 inhibition does not induce cell death in unstimulated pMφs



Supplementary Figure 1 | Inhibition of caspase-1 or NLRP3 inflammasome activation does not induce cell death in unstimulated pMφs.

Total PECs from *Gata6*-WT or *Gata6*-KO^{mye} mice were either unstimulated or stimulated with 100 ng ml⁻¹ LPS in the presence of DMSO (vehicle), 40 μM YVAD, or 1 μM MCC950 *ex vivo* for 16 hours. The percentage positive PI⁺ pMφs (gated by CD11b⁺F4/80⁺) was determined by flow cytometry. Data is from one experiment.

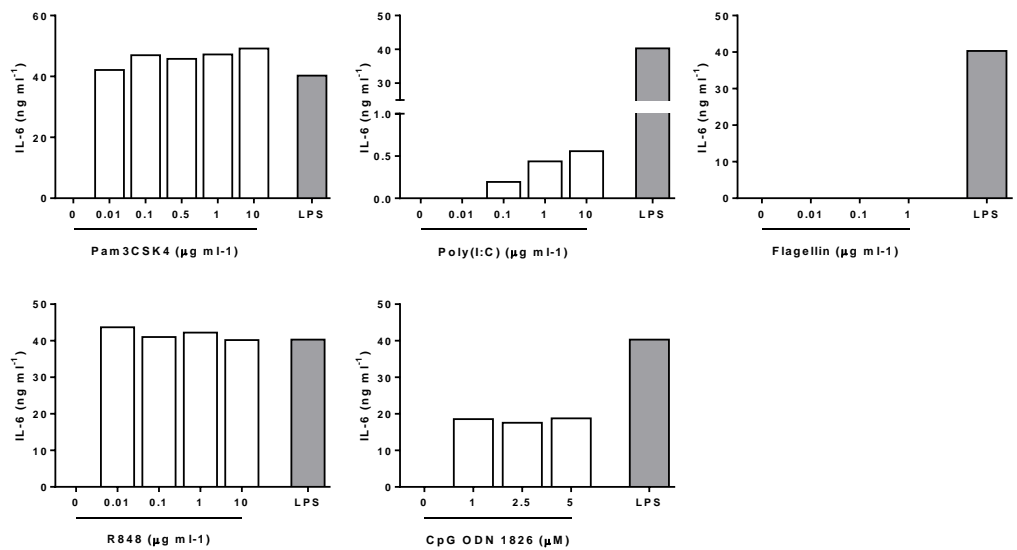
Appendix B | TLR expression in pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice



Supplementary Figure 2 | TLR expression in pMφs.

Microarray expression of genes encoding TLR receptors in pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice. Data is expressed as mean \pm S.D from three biological replicates. Statistical significance was determined using an empirical Bayesian statistic corrected for false discovery rate by the Benjamin-Hochberg procedure (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

Appendix C | IL-6 secretion from *Gata6*-WT pMφs after stimulation with various TLR ligands at different concentrations

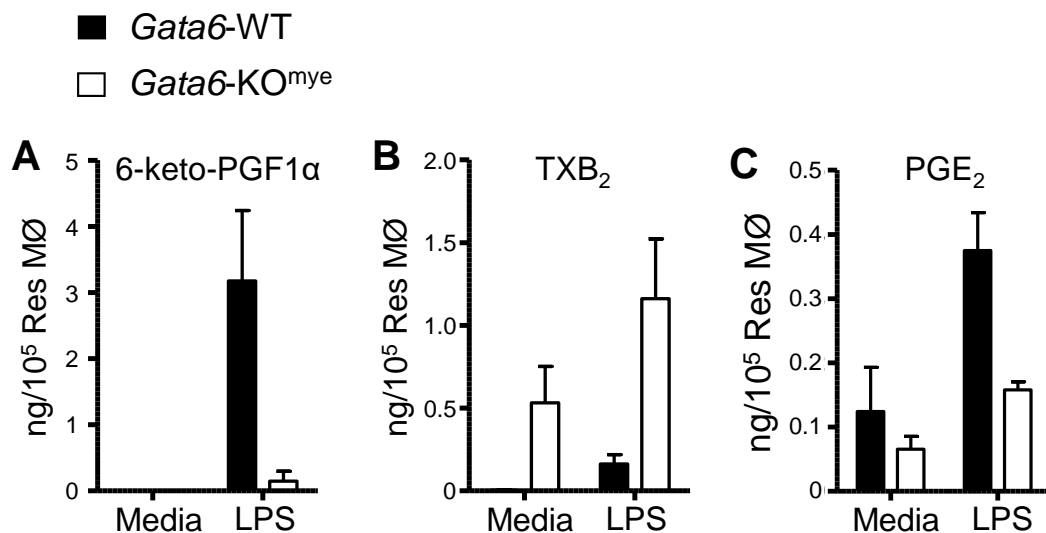


Supplementary Figure 3 | IL-6 secretion from pMφs from *Gata6*-WT mice in response to different TLR ligands.

APECs from *Gata6*-WT mice were stimulated with the indicated TLR ligands at different concentrations for 24 hours. Supernatants were collected and analysed by ELISA for IL-6 release. Data is from one experiment.

Appendix D | Prostanoid production in *Gata6*-deficient pMφs

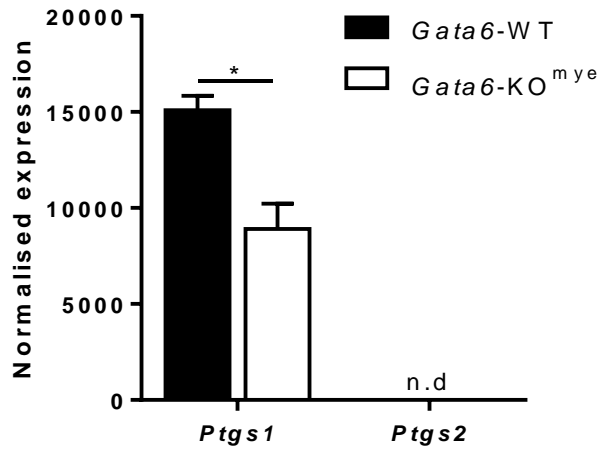
The loss of *Ptgis* expression in *Gata6*-deficient pMφs is likely to affect the prostanoid profile produced in response to microbial ligands, which in turn will specify the inflammatory response. To address this, Dr Marcela Rosas analysed the major prostanoids produced by unstimulated and LPS-stimulated pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice by mass spectrometry, which showed significant changes in the lipid profile (**Supplementary Figure 4**). In accordance with the significant decrease in *Ptgis* expression, the production of PGI₂ was significantly attenuated in LPS-stimulated *Gata6*-deficient pMφs (**Supplementary Figure 4**). Furthermore, the production of PGE₂ was lower in *Gata6*-deficient pMφs stimulated with LPS. Conversely, the production of TXB₂ was significantly increased in LPS-stimulated *Gata6*-deficient pMφs.



Supplementary Figure 4 | PGI₂ is significantly reduced in LPS-stimulated *Gata6*-deficient pMφs and TXB₂ increased.

pMφs from *Gata6*-WT and *Gata6*-KO^{mye} were either unstimulated or stimulated with 100 ng ml⁻¹ LPS. Supernatants were analysed by mass spectrometry for (A) 6-keto-PGF1α, (B) TXB₂, and (C) PGE₂ release. This experiment was performed by Dr Marcela Rosas and is included because of its relevance.

Appendix E | *Ptgs1* expression is significantly reduced in *Gata6*-deficient pMφs



Supplementary Figure 5 | *Ptgs1* and *Ptgs2* expression in pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice.

Microarray expression of *Ptgs1* and *Ptgs2* in pMφs from *Gata6*-WT and *Gata6*-KO^{mye} mice. Data is expressed as mean \pm S.D from three biological replicates. Statistical significance was determined using an empirical Bayesian statistic corrected for false discovery rate by the Benjamin-Hochberg procedure (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).